EPSTEIN BARR VIRUS RELATED DIFFUSE LARGE B CELL LYMPHOMA OF THE ELDERLY- ASSESSMENT OF FREQUENCY IN AN INDIAN TERTIARY CARE CENTRE AND COMPARISON OF THE MORPHOLOGICAL FEATURES OF THE EBER-ISH POSITIVE AND EBER-ISH NEGATIVE CASES

A DISSERTATION SUBMITTED IN PART FULFILLMENT OF THE REQUIREMENTS FOR THE M.D. DEGREE BRANCH III (PATHOLOGY) EXAMINATION OF THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY, CHENNAI TO BE HELD IN APRIL 2017.

CERTIFICATE

This is to certify that this dissertation titled "**EPSTEIN BARR VIRUS RELATED DIFFUSE LARGE B CELL LYMPHOMA OF THE ELDERLY- ASSESSMENT OF FREQUENCY IN AN INDIAN TERTIARY CARE CENTRE AND COMPARISON OF THE MORPHOLOGICAL FEATURES OF THE EBER-ISH POSITIVE AND EBER-ISH NEGATIVE CASES**" is a bonafide work done by Dr. Ananthvikas J, in part fulfilment of the rules and regulations for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R Medical University, to be held in April 2017.

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The candidate has independently reviewed the literature, performed the data collection, analysed the methodology and carried out the evaluation towards completion of the thesis.

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CERTIFICATE

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Last, but not the least, I dedicate this work to all the patients, without whose clinical material, this would not have been a reality

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RATIONALE FOR THE STUDY

Diffuse Large B cell lymphoma (DLBCL) is one of the commonest B Cell Non-Hodgkin lymphomas seen in routine practice. This is a high-grade lymphoma requiring chemotherapy and follow-up of patients. A subset of these lymphomas was found to be associated pathogenetically with the Epstein Barr virus (EBV) and it was further demonstrated that cases of DLBCL who were positive for markers of EBV showed poor response to conventional chemotherapy and a more aggressive course of illness. Awareness of the EBV status of DLBCLs in high-risk groups such as elderly patients could aid in prognostication of such cases, avoiding unnecessary and toxic chemotherapy.

Currently available immunohistochemical markers for EBV include EBV Latent Membrane Protein 1 (LMP1), which although commonly used, has its own limitations. The Latent Membrane Protein 1 is expressed in only two of the three latency phases of EBV, and interpretation could be challenging owing to the patchy, cytoplasmic staining characteristics.

Epstein Barr Encoded RNA (EBER) In Situ Hybridisation (ISH) is a newer technique that helps improve detection of EBV RNA in tissue sections. EBER is expressed in all 3 latency stages of EBV. The positive signals are stained blue on a red background, aiding in easy identification of positives.

Available literature on the incidence of EBV positive DLBCL (EBV+DLBCL) in elderly persons is predominantly from the western world and Europe, with a few reports from Korea, Japan and China. Currently, there is no data on the incidence of these EBV positive DLBCLs in India.

AIM AND OBJECTIVES

AIM

To assess the disease frequency of EBV positive cases of nodal diffuse large B cell lymphoma in our setting diagnosed using the Epstein Barr Encoded RNA In Situ Hybridisation (EBER-ISH) technique and comparison of the morphology of EBER positive and EBER negative cases.

OBJECTIVES

1. To identify cases of EBV positive diffuse large B cell lymphoma in patients older than 45 years of age in our institution during the period of January 2009 to September 2014 using the Epstein Barr Encoded RNA In Situ Hybridisation technique on lymph node biopsy specimens.
2. To assess the disease frequency of nodal EBV positive diffuse large B cell lymphoma in patients older than 45 years in our setting.
3. To do a detailed histological study of all cases and identify morphological features that could be useful in suspecting EBV positivity.

HYPOTHESIS

The frequency of EBV positive diffuse large B cell lymphoma in patients older than 45 years is higher in our setting than in the western population.

REVIEW OF LITERATURE

Introduction

EBV positive diffuse large B cell lymphoma (EBV+DLBCL) of the elderly has been considered as a provisional entity in the "World Health Organization classification of tumours of haematopoietic and lymphoid tissues", and has been defined as "EBV-positive clonal B-cell lymphoid proliferation that occurs in patients >50 years and without any known immunodeficiency or prior lymphoma".(1) The age limit of >50 years may have been considered as these patients, owing to age related senescence of the immune system, tend to have defective immune surveillance for the Epstein Barr Virus (EBV). Initial reports suggested a predominant extra-nodal occurrence of this neoplasm (stomach, lung, skin, tonsils) with additional lymph node involvement, whereas only around 1/3rd of cases were found to involve the lymph nodes alone. Known cases of immunodeficiency including causes secondary to immunosuppression, autoimmune diseases and transplantation need to be excluded for a diagnosis of this entity.

Epstein-Barr virus infects most people in childhood or adolescence, and usually leads to adaptive immunity. Infection occurring in adolescence or young adulthood leads to infectious mononucleosis in 35%-69% of cases. Lymphoproliferative disorders associated with EBV include B-cell lymphomas, T/NK cell lymphomas and HIV related lymphoproliferative disorders as follows.(2)

- EBV associated B cell lymphoproliferative disorders
    - Hodgkin lymphoma

    - Burkitt lymphoma
    - Post-transplant lymphoproliferative disorders
    - Lymphomatoid granulomatosis
    - Pyothorax associated lymphoma
    - Senile EBV associated EBV positive lymphoproliferative disorders
- EBV associated T/NK-cell lymphoproliferative disorders
    - Angioimmunoblastic T cell lymphoma
    - Extranodal nasal T/NK-cell lymphoma
    - Non Hepatosplenic γδ T-cell lymphomas
    - Enteropathy-type T-cell lymphoma
    - Peripheral T-cell lymphoma
- EBV associated HIV-related lymphoproliferative disorders
    - Primary CNS lymphoma
    - Primary effusion lymphoma
    - Plasmablastic lymphoma

Post-transplant lymphoproliferative disorders (PTLD) are a serious complication following solid organ transplantation. Majority of these cases are associated with EBV, and could be linked to the re-activation of dormant EBV owing to post transplant immunosuppression. In children, however, a majority of cases are linked

to primary EBV infection occurring post-transplant.(3) Recently, Gibson et al described "EBV positive Extranodal Marginal Zone Lymphoma of Mucosa Associated Lymphoid Tissue" in the post-transplant setting as a distinct entity.(4) According to the 2016 edition of the WHO classification of lymphoid neoplasms, post-transplant lymphoproliferative disorders include Plasmacytic hyperplasia PTLD, Infectious mononucleosis PTLD, Florid follicular hyperplasia PTLD, Polymorphic PTLD, Monomorphic PTLD and Classical Hodgkin lymphoma PTLD.(5)

Oyama et al, in 2003, published a paper titled "Senile EBV+ B-Cell Lymphoproliferative Disorders" in the American Journal of Surgical Pathology, where they evaluated twenty-two cases of Epstein-Barr virus associated B cell lymphoproliferative disorders with no predisposing immunodeficiency. These patients had a median age of 75.5 years and were all more than 60 years old, and 18 of these cases shows extra-nodal involvement. A few other studies on EBV+DLBCL have followed since, representing different geographic regions of the world and have shown a varying incidence of EBV positivity in cases of DLBCL of the elderly.(6–12)

Epidemiology:

The prevalence of EBV related DLBCL of the elderly is varied according to reports. According to a study by Lu et al from China published in Nature in 2015, an analysis

of EBER-ISH positivity in 250 cases of DLBCL revealed an incidence of 15.1% in the elderly group and 11.9% in the younger group when they considered a threshold for positivity as 20%. In contrast, when a threshold for positivity of 50% was applied, the incidence of EBER positivity reduced to 11.4% in the elderly group and 8.3% in the younger group.(13) Similarly, in a study from Peru by Beltran et al comprising 199 elderly patients of DLBCL, the incidence of EBER-ISH positivity was found to be slightly higher 14.9% and 9% when cut-off values of 20% and 50% respectively were applied.(6) Park et al, in a study on 380 cases of DLBCL from Korea, reported an incidence of 8.9% positivity with a cut-off of >20%.(14)

Studies from Japan have revealed varying results. A study by Kuze et al published in the Japanese Journal of Cancer Research in 2000 reported an incidence of 11.4%. However, no mention was made about the cut-off value considered for EBER ISH positivity.(7) Oyama et al in 2007 reported a similar incidence of 8.7% of EBV positive DLBCL when a cut off of 50% was applied.(8) On the other hand, in a study by Wada et al published in the Journal of Medical Virology in 2011, the incidence was found to be much lower at 3.3% and 1% when cut-off values of >20% and >50% respectively were applied, and this was found to be comparable to the incidence reported in the western hemisphere.(15)

A study published in Modern Pathology by Hofscheier et al compared cases of EBV positive DLBCL between Mexican and German populations and reported a higher incidence in the Mexican cohort (7%) when compared to the German cohort (2%) when a cut-off of >20% positivity for diagnosis was applied.(10) Similarly, Hoeller

et al identified a much lower incidence of 3.1% in the European population with the analysis being done using a tissue microarray method.(16)

Ok et al in 2014 studied the prevalence of EBV infection in cases of DLBCL occurring de novo in a large multicenter cohort of 732 patients from the USA, Europe, China and South Korea, and found EBV positivity in 28, i.e. 4% of patients, with a mean age of 60.5 years. However, they included all age groups in their study, possibly explaining the lower mean age when compared to other cohorts.(17)

Clinical features

EBV+DLBCL of the elderly affects people more than 50 years of age with a slight male preponderance.(18) In a study published by Park et al, they found EBV positivity in 9% of cases, which showed a strong association with age older than 60 years, advanced stage of disease, multiple extranodal sites of involvement, higher clinical international prognostication index, presence of B symptoms and a poorer response to therapy.(14) There seems to be no predilection for nodal or extranodal sites, with variable incidence reported in literature. Some of the common extranodal sites of involvement include skin, soft tissue, bones, stomach, tonsils, tongue, lung, pleura, liver, spleen, peritoneum and bone marrow. They generally tend to show an aggressive course of disease, and studies from Asia report a mean survival of 2 years.(6,8–10,14,15) When CD30 is also expressed, the survival rates in cases of EBV+DLBCL of the elderly has been found to be worse.

Dojcinov et al classified the spectrum of EBV positive B cell lymphoproliferative

diseases in adults into 4 diagnostic categories: 1) Reactive lymphoid hyperplasia; 2) Polymorphic extranodal lymphoproliferative disease; 3) Polymorphic nodal proliferative disease and 4) Diffuse Large B Cell Lymphoma. They found that the five-year survival decreased progressively in these groups, in contrast to the frequency of monoclonality that was found to increase.(11) Two recently described entities add to the spectrum of EBV positive lymphoproliferative disorders, including EBV positive mucocutaneous ulcer and Plasmablastic lymphoma of the elderly. EBV positive mucocutaneous ulcer is a term used to describe a small volume extranodal polymorphic lymphoproliferative state especially involving mucosal sites and skin, characterized by an indolent course of illness and a good prognosis.(19) Plasmablastic lymphoma of the elderly has also been studied to show an indolent course and overall better prognosis compared to other age related EBV positive B cell lymphoproliferative disorders.(20)

Etiopathogenesis:

The etiopathogenesis of EBV+DLBCL of the elderly has been postulated to be a related to immune senescence that occurs with aging. From an immunologic standpoint, aging results in a dysregulated relationship between inflammatory and inflammation neutralizing factors that results in a chronic low grade pro-inflammatory state that could lead to lymphomagenesis. This effect may be related to dysregulation of critical oncogenic pathways such as p53, retinoblastoma, nuclear factor kB and mitogen activated protein kinases by radical oxygen species. Immunosenescence also refers to a continuous remodeling process where the

adaptive immunity is preferentially affected compared to the innate immunity, with resultant decrease in B cell diversity with age and a concurrent clonal expansion of B cells in vivo. The T cell compartment also shows changes such as a reduction in the absolute number of total T lymphocytes including CD4+ and CD8+ cell counts, a progressive decrease in the naive T cell population with a decrease in the T cell diversity, and a progressive expansion of oligoclonal CD28- T cells, especially among the CD8+ T cell subset.(21,22)

There is growing evidence to suggest a clonal expansion of dysfunctional EBV specific cells to age leading to the shrinkage of the T cell repertoire available for novel antigens. A study by Ouyang et al evaluated the frequency of CD8+ T cells that carried the receptors for an immunodominant EBV lytic epitope in old and young patients by direct staining with HLA peptide tetrameric complexes. They found a significantly higher frequency of EBV tetramer positive cells within the CD8+ T cell subset in the old compared to the young, whereas the EBV antigen specific Interferon gamma producing T cells were less frequent.(23)

Epstein Barr Virus:

The Epstein Barr virus was the first human tumour virus to be isolated, and was identified by Epstein and colleagues in 1964 in a cell line derived from Burkitt lymphoma.(24) EBV is a gammaherpes virus considered as the prototype of the *Lymphocryptovirus* genus, and is formally designated as Human Herpesvirus 4 (HHV-4). Two major EBV types, EBV-1 and EBV-2, have been detected in humans,

that differ in the genetic sequence that codes for the EBV nuclear antigens.(25) The DNA sequence heterogeneity found when comparing specific regions of the EBV genome from different geographical locations could be explained by the two types containing different strains.(26)

EBV is a DNA virus and contains a linear double stranded 172kb DNA molecule that encodes more than 85 genes. The EBV open reading frames are named based on the BamH1 restriction fragment on which they are found. The many EBV open reading frames are further classified as latent and lytic genes, further divided into immediate early genes, early genes and late genes. Several of these lytic genes encode for human homologues, while some latent genes are non-translated. This is the case of EBV encoded RNA (EBER) 1 and 2.(27) The viral genome also contains a series of 0.5 kb terminal repeats at either end and internal repeat sequences that serve to divide the genome into short and long unique sequence domains that have most of the coding capacity.(28) These terminal repeats are good markers of whether EBV infected cells are from the same progenitor, as the viral DNA circularizes when it infects a cell and persists as a circular episome with a particular number of terminal repeats that depends on the number of terminal repeats in the parent genome.(29)

Humans are the only natural host for EBV. EBV, like other gamma-herpesviruses, can establish latent infection in lymphocytes and cause these latently infected cells to proliferate.(30) EBV infection of B cells is mediated through the interaction of viral envelope glycoprotein gp350/220 with the cellular receptor for C3d complement component CR2 (CD21).(31) Once the virus particle binds to the surface

of the host cell, it undergoes endocytosis and the viral envelope fuses with the host cell membrane. This process involves viral glycoproteins gp85, gp25 and gp42. Gp42 can bind to MHC class 2 and this is used as a cofactor by EBV in the infection of B lymphocytes,(32)

CD21 independent pathways may be responsible for the EBV infection of cells other than B lymphocytes such as T lymphoma cells and other epithelial cells including those seen in nasopharyngeal carcinoma, gastric carcinoma and oral hairy leukoplakia.(33) Current evidence suggests that EBV infection in healthy chronic virus carriers is restricted to B lymphocytes, and sometimes in epithelial cells that probably serve as sites for replication and amplification of EBV rather than a site for persistent latent infection. EBV survives in the host cell by establishing latency as an episome in memory B cells, and uses only a limited number of genes to maintain its genome and evade host reaction.

The Epstein Barr virus spreads via the saliva and enters the epithelium of the Waldeyers ring surrounding the oropharynx and probably initiates a lytic infection leading to amplification of the virus. Naïve B cells in the underlying lymphoid tissue are then infected to become activated lymphoblasts using the growth transcription programme (Latency III). In this phase, the gene products expressed are EBNA -1, -2, -3A, -3B, -3C, LP, LMP1, LMP-2A, LMP-2B and EBERs. The default transcription or latency II functions mainly to differentiate the activated B cells into memory B cells and exit the germinal centre. The gene products expressed during this stage are EBNA-1, LMP1, LMP-2A and EBERs. Then, the latency transcription

programme, i.e., latency 0, begins in the memory B cell in which all viral protein expression is turned off and this allows lifetime persistence. When these cells occasionally divide, they express the EBNA-1 only latency, i.e. latency 1. The memory B cells on returning to the tonsil may occasionally undergo plasma cell differentiation triggering viral replication. The resulting virus particles may be released into the saliva and further transferred to other hosts or infect other B cells. The different EBV latencies and their associated expression of viral products has been summarized below.(34)

Table 1: EBV latencies and their associated viral products

	EBER	EBNA-1	EBNA-2	EBNA-3	LMP-1	LMP-2
Latency I	+	+	-	-	-	-
Latency II	+	+	-	-	+	+
Latency III	+	+	+	+	+	+

In all forms of latency, EBV expresses two classes of non-coding small RNA (EBER 1 & 2) that are highly structures RNAs containing 167 and 172 nucleotides respectively. The expression of these is restricted to the cell nucleus where they are present at approximately 10^7 copies per cell. Further, specific latency EBV-transcription programmes have been demonstrated in many tumour. Type III latency is observed in patients with infectious mononucleosis or a subset of post-transplant lymphoproliferative disorders. Type II latency is commonly seen in classical Hodgkin lymphoma and a subset of post-transplant lymphoproliferative disorder.

Type I latency is characteristically seen in Burkitt lymphoma.(35) All these tumours probably originate in relation to specific stages in the EBV life cycle and are associated with disturbances of the immune system.(36,37)

The prevalence of EBV is high throughout the world, as suggested by numerous reports, and the exposure to EBV is likely to be linked to socioeconomic factors.(38,39) Two major types of EBV have been identified, i.e., EBV 1 and EBV 2, and differ in their geographic distribution, although the role of specific EBV types in the etiology of cancer is unknown. Immunocompromised patients generally tend to harbor both subtypes. The virus is generally transmitted by the oral route although other routes such as through transfusion have been reported.

Following primary infection by transmission of cell free virus or infected cells via saliva, the virus enters the circulating B cell pool and escapes detection in most cases. The mucosal epithelial cells may also provide sites for intermittent viral replication. However, the resting memory B cells may be the true reservoir of infection, and also express a very restricted pattern of latent viral gene expression. Primary infection invariably leads to infectious mononucleosis. EBV related malignancies may arise in the background of viral reactivation by other cofactors.

Detection of EBV in virus associated malignancies could either be performed on the tissue sections or in serum. Cell free EBV DNA has been detected in the serum of patients with many malignancies including Hodgkin lymphoma, post-transplant lymphoproliferative disease, NK/T cell lymphoma, Burkitt lymphoma and

nasopharyngeal carcinoma, but has not been detected in healthy carrier controls. This indicates that EBV DNA is not found in the serum in the absence of active EBV disease. Detection of plasma EBV DNA by PCR has been proposed as a sensitive and specific diagnostic marker for tumour diagnosis.

Detection of EBV in tumour cells can be performed by PCR or in-situ hybridization using the BamH1 internal fragment of the viral genome as a probe. Recently however, the use of probes specific for small nuclear EBV encoded RNAs, EBER-1 and EBER-2 which are expressed in all types of EBV infection has provided a major breakthrough in routine diagnosis with a high sensitivity. In cases of nasopharyngeal carcinoma, detection of EBV genomic DNA shows high loads.

EBV expresses 3 latent membrane proteins during the periods of latency II and III, i.e., LMP 1, LMP 2A and LMP 2B. LMP 2A can also be expressed in resting virus carrying B lymphoblasts in healthy individuals, representing the reservoir of latent infection.(40) LMP 2A in combination with LMP 1 are necessary for continued lymphoma survival via the TRAF regulation of Nuclear Factor kB.(41) The Latent membrane proteins are multifunctional and are expressed on the cell membrane as well as intracellular membranes of the Golgi and endoplasmic reticulum.

Latent membrane protein 1 (LMP1) although essential is not mandatory for the transformation of B lymphocytes into lymphoblastoid cell lines, and EBV mutants lacking LMP 1 fail to effectively immortalize B cells. LMP 1 is an integral membrane protein and acts as a constitutively activated receptor and is functionally similar to

the tumour necrosis factor receptor family of proteins It also leads to activation of nuclear factor kB, JNK kinase and JAK/STAT pathways, with a downstream effect of protection from apoptosis. LMP 1 causes upregulation of anti-apoptotic proteins such as A20 and Bcl2 and can block apoptosis. LMP 1, when expressed on epithelial cell in vitro blocks DNA repair and induces micronuclei formation, chromosomal aberrations and genomic instability.(42)

Latent Membrane Protein 2A (LMP 2A) in vitro when studied on transgenic mice has been found to promote the survival of mature B cells in the absence of surface immunoglobulin expression. LMP 2A is essential for growth transformation of B cells as EBV mutants that lack the LMP 2A fail to allow germinal centre cells to survive. Studies on lymphoblastoid cell lines have shown that LMP 2A blocks both B cell receptor signaling and antigen processing functions.(43) LMP 2A has been suggested to have an important role in maintaining viral latency. It has also been found to modulate cell growth and apoptosis by activation of PI3 Kinase. LMP 2A can also potentially inhibit the activation of lytic EBV replication by cell surface mediated signal transduction.

The EBV-encoded RNAs (EBERs) are 2 non coding and non polyadenylated RNAs that are always expressed in large numbers in latently infected cells irrespective of the phenotype. These act as regulators of signaling and transcription factors resulting in production of interferons and cytokines. They are also found to induce Interleukin 10 as an autocrine growth factor in Burkitt lymphoma via retinoic acid inducible gene I mediated activation of IRF 3. EBER-2 via its induction of interleukin 6 has also

been thought to contribute to B cell transformation.

Programmed Death Ligand 1 (PD-L1) has been studied in certain virus and immunodeficiency associated lymphomas. PD-L1 is an immunomodulatory molecule expressed by antigen presenting cells and a few tumour cells, and engages receptors on T cells to inhibit T-cell mediated immunity. Clinical trials on anti PD-L1 antibodies have shown long lasting clinical response in patient with solid tumours.(44,45) PD-L1 has been shown to be expressed by Reed Sternberg cells of Classical Hodgkin lymphoma and the malignant B cells in EBV positive Post Transplant Lymphoproliferative Disorder.(46) Chen et al in 2013 demonstrated robust and strong PD-L1 expression in most cases of nodular sclerosis and mixed cellularity Hodgkin lymphoma, EBV positive post-transplant lymphoproliferative disorders, T cell rich large B cell lymphoma, EBV positive diffuse large B cell lymphoma, nasopharyngeal carcinoma, extranodal NK/T-cell lymphoma and HHV-8 associated primary effusion lymphoma.(47)

Epstein Barr Virus and Diffuse Large B Cell Lymphoma

The pathogenesis of EBV+DLBCL is now understood to be a combination of immune senescence that further enables the proliferation and outgrowth of EBV infected B cell clones. This is further supported by the morphologic similarities between EBV+DLBCL of the elderly and immunodeficiency associated lymphoproliferative disorders such as PTLDs. Subclinical expansion of EBV

positive B cells may preclude the onset of over disease.(48)

A study by Fagnoni et al provided further insight and evidence toward the age related senescence of the immune system. They studied the circulating naïve CD95 negative T cell reservoir throughout the human lifespan, based on the fact that the ability to mount a primary immune response rests on these cells. They found a sharp decrease in the naïve T cells with age, and the oldest individuals were almost completely depleted of circulating CD28 positive cells.(49) The combination of decrease in naïve T cells and reduction of CD8+ T cell function(23) in an exhausted immune system seen in the elderly may facilitate the uncontrolled proliferation of EBV positive B cells, and may provide an explanation to increased incidence and severity of disease in the elderly.

In a study by Oyama et al, they found 32% of the cases to express EBNA2, therefore displaying a type III latency. This indicates a reduced immunity against EBV and is believed to occur only in the setting of an immunodeficiency with reduced antibody response to EBNA 1, EBNA 2 and EBNA-LP.(50) There was no prognostic difference however, between cases displaying latency type II or latency type III. It has been presumed that cases positive for EBNA 2 may have an additional cause of immunosuppression rather than just age related Immunosenescence.

However, recent studies have brought to light the occurrence of EBV positive DLBCLs in younger patients as well. A study by Lu et al found that over a follow up period of 29 months, patients with EBV+DLBCL showed an overall survival that

was significantly worse in both the older and younger age groups. Further, the older and younger groups showed a similar overall survival and progression free survival with no significant difference between the two groups.(13) This was in contrast to another study by Hong et al who reported that EBV positive DLBCL in young adults was not associated with unfavorable outcomes.(51) An elegant study by Nicolae et al published in 2015 described 46 cases of EBV+DLBCL in patients younger than 45 years of age. They discussed that the EBV+DLBCL in the young resembles those of the elderly in many respects, in that they both expressed a predominant activated B cell or Non Germinal B cell phenotype and had an EBV latency type II. However, in contrast to the elderly type, EBV+DLBCL of the young showed a clinical response to standard treatment protocols and showed a favorable outcome. They also found a higher proportion of nodal disease rather than extranodal disease, which is in contrast to that observed in EBV+DLBCL of the elderly.(52)

These findings led to the questioning of the arbitrary age cut off of >50 years as defined by the WHO in the 2008 classification of lymphoid neoplasms. Ok et al studied EBV positive DLBCL in a Caucasian population and found a similar clinocopathologic, immunophenotypic and genetic profile in patients <50 years and >50 years of age. However, they found a poorer performance status in the elderly group. They proposed that the arbitrary cut off of 50 years for EBV positive DLBCL is unnecessary, and this has further been considered for implementation in the latest 2016 edition of the WHO classification of hematopoietic and lymphoid neoplasms.(5,53)

Morphology of EBV positive DLBCL:

Histologically, EBV positive DLBCL may present as two subtypes- Polymorphic and monomorphic.(54) The polymorphic subtype is more common and is shows B cells in varying stages of maturation including centroblasts, immunoblasts and plasmablasts with a background infiltrate of small lymphocytes, plasma cells and histiocytes. Reed Sternberg cells may sometimes be seen in the infiltrate, and may sometimes be prominent enough to pose a diagnostic difficulty with Hodgkin lymphoma. The monomorphic subtype on the other hand shows sheets of large transformed B cells. This distinction, however interesting, is not considered clinically significant.

Other histologic features reported include large areas of geographic necrosis or apoptosis, both of which are considered to be important and raise a suspicion of EBV positive DLBCL.(18) The pattern of infiltration could vary from nodular to vaguely nodular and diffuse. Moreno et al reported that the predominant subtype of DLBCL in EBV positive cases is of the non-germinal centre/ activated B cell type.(9) In contrast, Park et al reported a similar proportion of Germinal centre B cell subtype in both EBER positive and EBER negative cases.(14)

Nicolae et al in their study of EBV+DLBCL in patients <50 years of age recognized 3 main histomorphologic patterns including T-cell/histiocyte rich large B cell lymphoma like, B cell lymphoma unclassifiable with features intermediate between DLBCL and Classical Hodgkin Lymphoma (Grey zone lymphoma), and DLBCL-

NOS. They also observed a higher frequency of CD30 positivity and PD-L1 expression in the younger patients when compared to the elderly, with most cases exhibiting a non-germinal center B cell phenotype.

Immunophenotype of EBV positive DLBCL

By definition, EBV+DLBCLs show positivity for B cell antigens including CD20 and/or CD19, CD22, CD79a, PAX5, and are negative for pan T cell markers. Plasmacytoid variants may show a weak positive of a negative staining for CD20. Immunoglobulin light chain restriction may not be demonstrable except in cases with immunoblastic or plasmablastic morphology where cytoplasmic immunoglobulin can be assessed. The activated B cell (ABC) phenotype is more common and these cases may show positivity for MUM/IRF4 and negative for CD10 and Bcl6. CD30 may be positive, however CD15 is usually negative.(9,18) The Ki-67 proliferation index is invariably high. The neoplastic cells by definition express EBER, and variably express LMP1 (>90%) and EBNA2 (15-30%).(55) EBER and LMP1 show 80%-90% concordance but are not uniformly consistent.(35) The cut-off for EBER positivity has not yet been clearly defined by the WHO, and many investigators have applied cut off values ranging from 20% to as high as 80%, with the commonest value being 50%.

The proportion of EBV positivity among all cases of DLBCL increased with age, and this is similar to that of the ABC subtype. In view of this, it is speculated that there is either a change in B cell population with aging or there is a presumed pathological specificity of EBV in elderly patients with DLBCL.(56)

Molecular analysis

The data currently available on molecular analysis of EBV positive DLBCL is limited. Most studies have shown a monoclonal rearrangement of Immunoglobulin Heavy gene (IGH).(12,50) Adam et al demonstrated B cell monoclonality in 73% of EBV positive DLBCL, which could be used in differentiation from cases with reactive atypical EBV positive lymphoproliferations in the elderly. The very little literature available on EBV genotype suggests an equal distribution of EBV type A and EBV type B among cases of DLBCL.(55) A majority of the cases of EBV+DLBCL of the elderly have demonstrated an activated B cell phenotype. Recently, it has been demonstrated that NF-κB activity is elevated in both the activated B cell and the Germinal centre B ell subtypes, pointing towards a potential use of NF-κB inhibitory therapy.(9)

In recent times, numerous hot spot regions in the B cell receptor and Toll like receptor mediated NF-κB signaling have been found in cases with the activated B cell phenotype, while mutations of the EZH2 gene have been found in association with the germinal center B cell type of cases, leading to the development of targeted therapy in subsets of DLBCL. An interesting finding in a study by Gebauer et al was a slight predominance of the germinal center B cell subtype of DLBCL over the activated B cell subtype. They proposed that "the Hans algorithm that is widely employed to classify DLBCL based on immunohistochemistry, is likely to be biased in cases harboring an EBV infection owing to the EBV nuclear antigen 3C driven deregulation of IRF-4 (Mum-1)".(57) They further proposed that EBV infection

could lead to direct amplification of NF-κB activation through EBV LMP1 and LMP2A in both non-neoplastic and malignant B cells (such as in DLBCL).(9,58,59)

Differential diagnoses:

The spectrum of differential diagnosis of EBV positive DLBCL ranges from benign to malignant conditions. EBV reactivation leading to an infectious mononucleosis like illness in old age must be considered. The EBV associated reactive lymphoid proliferations are generally characterized by an expansion of the inter-follicular area with proliferation of small blood vessels. There is usually a mixed infiltrate of small lymphocytes, plasma cells and immunoblasts, some of which resemble Reed Sternberg cells. EBER in situ hybridization may show scattered positive cells in the interfollicular area.

EBV positive Classical Hodgkin Lymphoma occurring in older patients is one of the most challenging differential diagnosis of EBV+DLBCL. The strong homogenous expression of CD20 in more than 50% of Reed Sternberg like cells, along with expression of B cell specific transcription factors like OCT-2 and BOB 1 favor a diagnosis of EBV+DLBCL over a Classical Hodgkin lymphoma.(60) The expression of CD30 may not be useful in differentiation. EBER and EBV LMP1 are invariably expressed in cases of Classical Hodgkin lymphoma of the elderly as they are more often associated with EBV. The elevated number of cytotoxic T cells positive for TIA1 and/or Granzyme B, amounting to more than 30% of the background infiltrate is also a feature in favor of EBV+DLBCL of the elderly.(54)

Plasmablastic lymphoma invariably manifests in extranodal sites, and is more often secondary to immunodeficiency states. Morphologically, it is characterized by a diffuse proliferation of large cells morphologically resembling centroblasts and plasmablasts but immunophenotypically resembling plasma cells. Tumour cells are usually negative for CD20 and variably express IRF4/MUM1, CD138 or CD 79a.

MATERIALS AND METHODS

Introduction

This study was performed in the department of general pathology, Christian Medical College, Vellore. Cases were retrieved from the Oracle based electronic database using a key-word search. It was observed that approximately 50% of cases were of DLBCL involving the lymph node, among which there was an almost equal proportion of cases less than 45 years of age and more than 45 years of age. We adopted an age criteria of 45 years based on available literature.(52,53,61,62)
A literature search was then performed for EBV positive DLBCL of the elderly, and the reported incidence in each study was noted. The statistician then helped us calculate a sample size of 138 cases with a 95% confidence level that was based on the existing prevalence in published literature, the expected prevalence in our setting and the average number of cases reported in our department.

The institutional review board and ethics committee of the Christian Medical College, Vellore, approved this study (IRB Reference number 9144 dated 12-11-14).

All cases of lymph nodal DLBCL diagnosed between January 2009 and September 2014, in patients older than 45 years, were identified from the electronic database of the department of General pathology. The available clinical discharge summaries and laboratory investigations on the electronic hospital information system database were analysed and relevant clinical and serological data was collected.

Inclusion criteria:

- Cases of lymph nodal diffuse large B cell lymphoma occurring in patients older than 45 years of age

Exclusion criteria:

- Patients with a documented positive infectious serology test for any of the blood borne viruses
- Patients less than 45 years of age at the time of biopsy
- Patients who were either on treatment or had received treatment for lymphoma
- Cases of diffuse large B cell lymphoma with features suggestive of a grey zone lymphoma (features intermediate between DLBCL and Burkitt lymphoma) or a double hit lymphoma
- Cases with an initial presentation of DLBCL involving an extranodal site/organ with subsequent spread to lymph nodes
- If paraffin blocks were not available
- If paraffin blocks were available but contained very tiny or absent tissue fragments, insufficient for further evaluation

The first 129 consecutive cases of nodal DLBCL that fulfilled the above criteria were then selected for the study. The initial sample size of 138 was calculated assuming an incidence of 10% in our population with a 95% confidence level.

However, we were able to retrieve only 129 cases owing to unavailability of blocks or scanty tissue in the archived blocks. This final sample size of 129 was subsequently checked for power, which was found to be satisfactory at 99.99% and was hence considered adequate for the present study.

Clinical parameters:

Patient demographics: Patient demographics details including the age, gender and state of residence were noted

Site of biopsy: The site of lymph node biopsy was noted, including the presence of multiple lymph node involvement at presentation.

Clinical stage, B symptoms and IPI score: The clinical stage, presence or absence of B symptoms, and the international prognostic index (IPI) score for each patient as recorded in the discharge summaries or outpatient notes by the clinical hematologist were noted.

Presence of marrow involvement at presentation: In those cases, where a bone marrow trephine biopsy was done at presentation, the presence or absence of lymphoma involving the marrow was noted.

Histological study:

The available Hematoxylin and Eosin stained sections of 129 cases selected for the study were studied. Those slides that had poor preservation of staining or other artifacts were re-stained or re-mounted accordingly. The slides were initially reviewed for the ten histologic parameters by the candidate, and then further reviewed by both the candidate and the guide together on a double-header microscope. This combined review helped in eliminating observer bias.

The following were the histological parameters evaluated, and the observations were recorded in a Microsoft Excel® worksheet

Pattern: The architecture of the tumour as seen on a low power objective was broadly categorized as diffuse and vaguely nodular. Among the cases that showed a combination of these patterns, the major and minor patterns were noted accordingly. Any deviation from these patterns was noted accordingly.

Subtype: Cases of DLBCL were categorized based on morphology as per the "2008 WHO classification of tumours of hematopoietic and lymphoid tissues" into centroblastic, immunoblastic and anaplastic variants. Centroblastic pattern was defined as those cases with a mixed population of centroblasts and immunoblasts, where immunoblasts amounted to <90% of tumour cells. Among these cases, those

that showed a relatively higher proportion of immunoblasts, but not amounting to 90% were noted. The immunoblastic pattern was defined as those cases with a mixed population, where the immunoblasts predominated and amounted to >90% of tumour cells. The anaplastic variant of DLBCL was defined as those cases which showed a population of large to very large, round, oval or polygonal cells that displayed bizarre pleomorphic nuclei.

Reactive background: A reactive background was composed of the background non-tumoural lymphocytes and any inflammatory cells including neutrophils and eosinophils. The reactive background was graded as absent, mild/focal presence when restricted to a few high power fields, readily evident when it was easily visible at a 100x magnification and marked reactive background when it obscured the tumour cells.

Necrosis: Necrosis of tumour cells, either confluent, focal or involving individual cells, was noted, and was classified as absent, focal necrosis or individual cell necrosis when it was restricted to a few high power fields and readily identifiable foci or confluent when it was identifiable in many low power fields.

Tingible body macrophages: The presence or absence of tingible body macrophages between the tumour cells was noted, and categorized as absent, very few or focally present when limited to a few high power fields and readily apparent cells.

Multinucleate giant cells: The presence or absence of multinucleate giant cells was identified and classified as absent or present. Cases with presence of multinucleate giant cells were further sub-classified as those with and without a Hodgkin Reed-Sternberg cell like morphology.

Vascular proliferation: The presence or absence of vascular proliferation was noted and classified as absent, focal or mild when visible only in a few high power fields and readily apparent vascular proliferation.

Angioinvasion: The presence or absence of angioinvasion as defined by tumour cells infiltrating the wall of a vessel was noted.

Fibrosis: The presence and the degree of intratumoural fibrosis was assessed and classified as no fibrosis, focal or mild fibrosis when limited to a few high power fields and readily evident fibrosis when easily visible on low power examination.

Perinodal extension: Perinodal extension, as evidenced by the presence of tumour cells in the perinodal adipose tissue classified as absence and presence. Those cases where perinodal extension was not readily apparent on initial hematoxylin and eosin sections, but which was detected on CD20 immunohistochemistry, were also considered as positives.

Immunohistochemistry:

Most cases had been worked up for CD3, CD20 and MIB-1. The archived slides were retrieved and examined as described below. For cases where slides were unavailable, the details as recorded in the database were considered.

CD3: The proportion of CD3 positive reactive T cells in the background was noted and graded as absent, <30%, 30-60% and >60%.

CD20: The presence / absence of CD20 staining was noted.

MIB-1: The MIB-1 proliferation index was counted at 400x magnification and expressed as percentage of tumour cells staining positive. The highest proliferation index found is each case was noted.

EBV-LMP1: EBV LMP1 immunohistochemistry was available in 7 cases. The presence or absence of EBV LMP1 positivity among tumour cells in these cases was noted.

Epstein Barr Encoded RNA In Situ hybridization (EBER-ISH):

In situ hybridization for EBER was performed using commercially available reagents sourced from Ventana Medical systems Inc. These reagents principally comprised of the "INFORM EBER probe" and the "ISH iVIEW$_{Blue}$" detection kit. All tests were performed on a Roche Ventana Benchmark XT automated platform. The standard EBER staining protocol for in situ hybridization as recommended by Ventana medical systems was used. A brief summary of the protocol is provided in the annexures.

After checking the archived paraffin embedded blocks for adequacy of tissue, sections were cut on Poly-L-lysine coated slides. 3 sections were accommodated on each slide owing to financial constraints. For every batch of slides that were stained, a control was included which was a known case of EBV positive DLBCL with documented positivity for EBV-LMP1 immunohistochemistry.

All stained slides were examined by the candidate at first, and then by both the candidate and the guide together on a double-header microscope in order to eliminate observer bias. Each case was evaluated for percentage positivity and the pattern of staining for EBER-ISH.

Statistical analysis**:**

Frequencies were calculated for each of the clinical and morphologic parameters. A Chi Square test and Fisher's exact test were used to compare the features of EBER ISH positive and EBER ISH negative cases to assess for significance. All statistical analysis was performed using the SPSS Version 16.0 software for Windows.

RESULTS

A total of 796 cases of DLBCL were diagnosed in the department of pathology, CMC Vellore between January 2009 and September 2014. 246 out of these primarily involved the lymph node. 263 out of the 796 were from patients more than 45 years of age.158 cases satisfied our inclusion criteria of nodal DLBCL in patients more than 45 years. Of these, 129 cases were retained for this study after elimination of 29 cases, 28 of which had inadequate tissue on paraffin blocks for further in-situ hybridization studies, and one case that was seropositive. The clinical and microscopic features were analysed in 129 cases.

The EBER-in situ hybridization test was performed on 114 of these cases. 5 of these cases showed an overwhelming background artifactual staining and therefore could not be assessed. 7 cases were positive for EBER-ISH, with >20% of cells staining positive. 1 case showed a few positively staining cells, ranging between 5-10%. 8 cases showed very occasional positively staining cells. Positivity for EBER-ISH in our study was considered as >10% tumour cells staining positive.

Frequency of EBV positive DLBCL in our population:

EBER-ISH staining was performed on 109 of the 129 cases, and was found to be positive in 7 of these. The calculated frequency of EBV+DLBCL in patients older than 45 years of age in our study was 6.42%.

Clinical features**:**

Age: The mean age at diagnosis of the cases of lymph nodal DLBCL in our study was 60.5 years, with the youngest patient being 47 years of age (Range 47-83 years)

Among the 7 cases that were positive or EBER-ISH, the mean and median age were found to be 65.8 years and 63 years (Range 50-82 years) in contrast to those cases negative for EBER-ISH that showed a slightly lower mean and median age of 60.2 and 59 years (Range 47-83 years).

Table 2: Age characteristics of EBER positive and EBER negative cases

Variable	*Mean*	*Median*	*Minimum*	*Maximum*
EBER positive	65.85714	63	50	82
EBER negative	60.53922	61	47	83

* EBER- Epstein Barr Encoded RNA

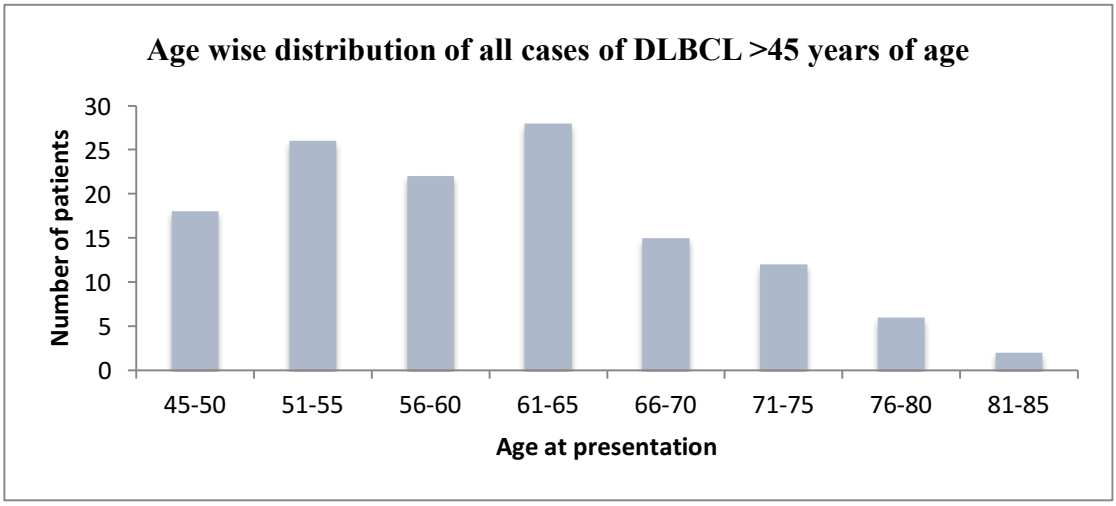


Figure 1: age wise distribution of cases of Diffuse Large B cell lymphoma older than 45 years of age

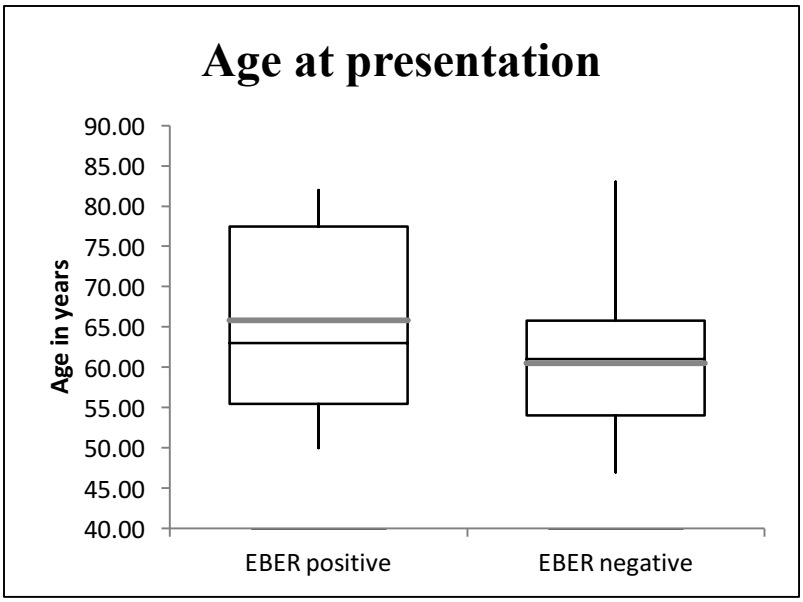


Figure 2: Comparison of the age at presentation of EBER positive and EBER negative cases

Gender: Overall, cases of lymph nodal DLBCL showed a male preponderance (2.07:1). All 7 cases that showed positivity for EBER-ISH were males.

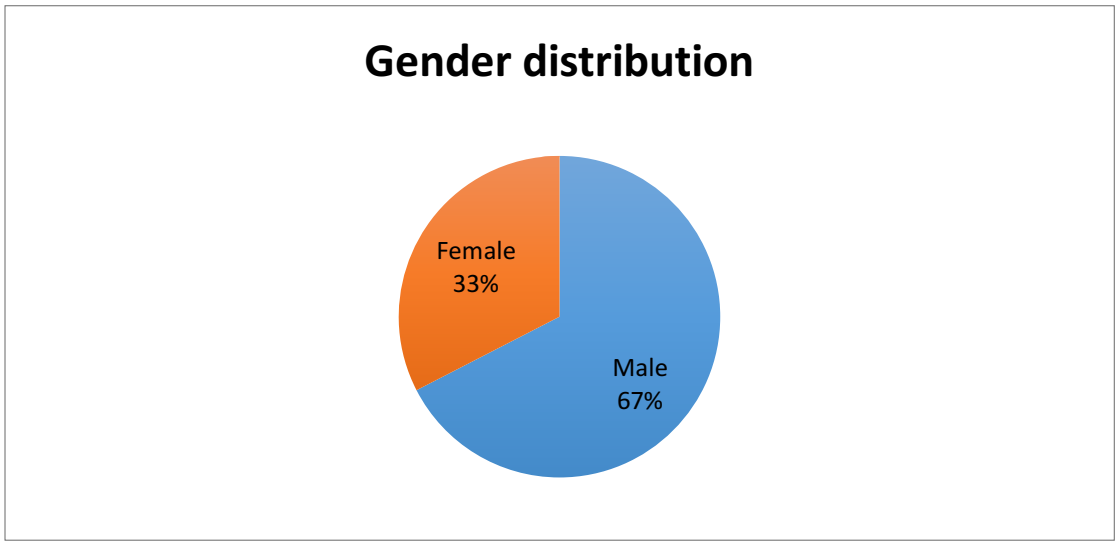


Figure 3: Gender distribution of all cases of nodal DLBCL >45 years of age

Patient demographics: The following chart depicts the geographical distribution of all cases evaluated in this study. The distribution of cases of DLBCL in our study reflected the demographics of patients who visit our hospital. There was no geographical predilection for EBV positive DLBCL seen in our study.

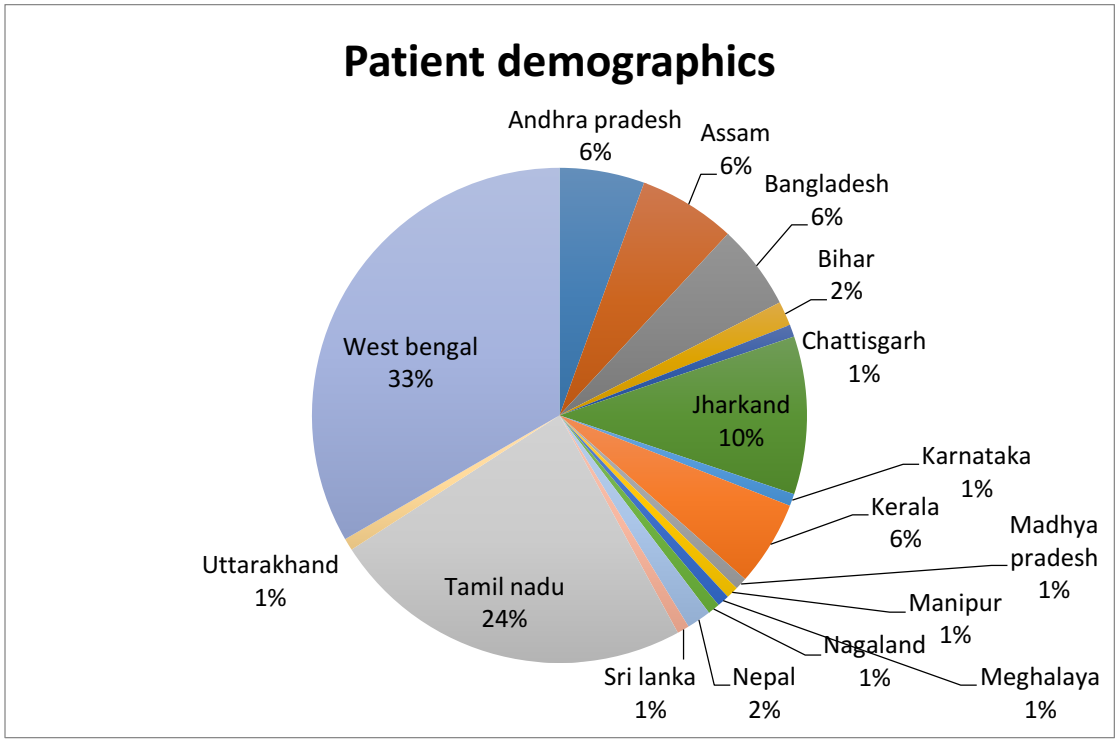
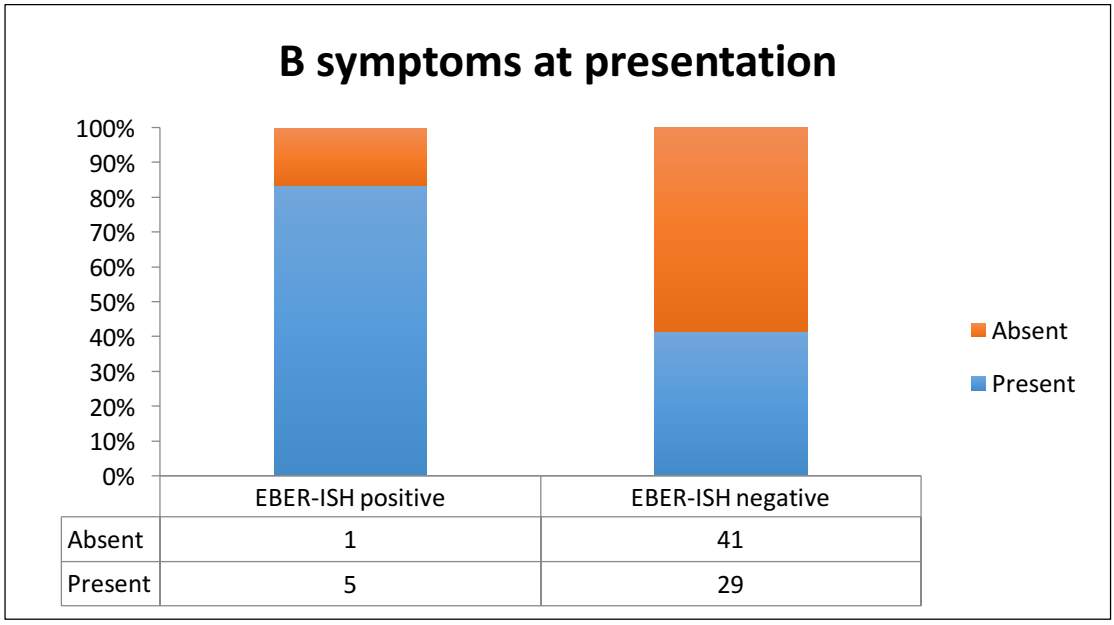


Figure 4: Patient demographics for all cases of nodal DLBCL >45 years of age

B symptoms: 76 cases had documented presence/absence of B symptoms in the patient records; including 6 EBER-ISH positive cases and 70 EBER-ISH negative cases. 5 out of the 6 (83.3%) EBER-ISH positive cases had B symptoms at presentation in contrast to 29 of 70 (58.5%) EBER-ISH negative with B symptoms at presentation.



	EBER-ISH positive	EBER-ISH negative
Absent	1	41
Present	5	29

Figure 5: Comparison of B symptoms at presentation between EBER-ISH positive and negative cases

Clinical stage: On review of the discharge summaries and out patient records, 61 out of the total 129 cases had documented clinical stage, including 6 out of 7 EBER-ISH positive cases and 55 of 102 EBER-ISH negative cases. Three cases each of EBER-ISH positive DLBCL were stage III and stage IV clinically.

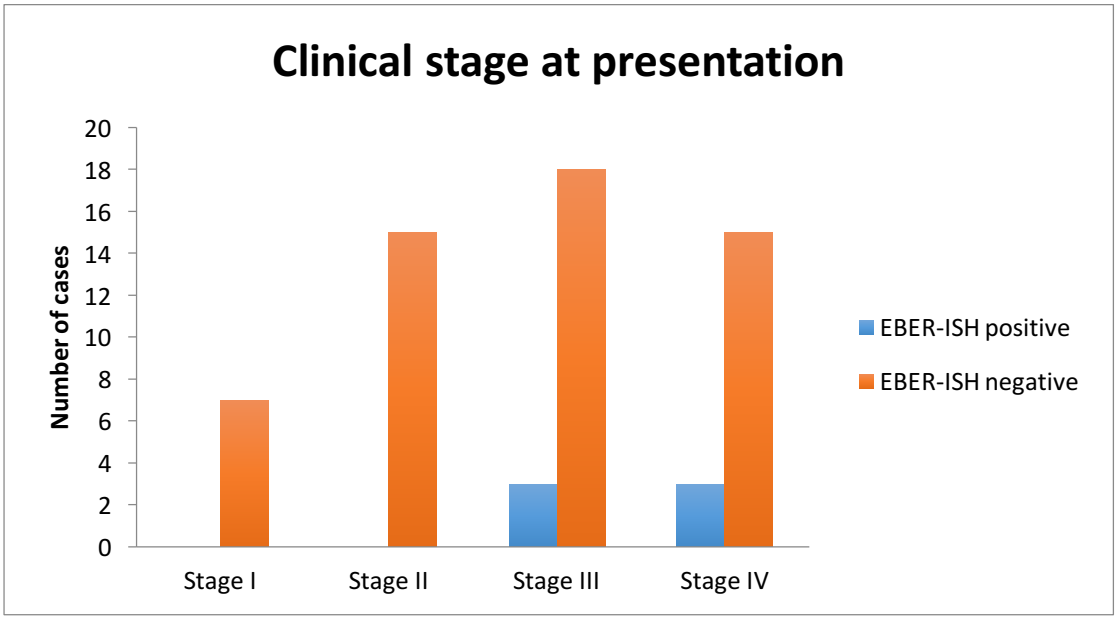


Figure 6: Clinical stage at presentation

International prognostication index (IPI) score: 38 of the total 129 cases had a documented IPI score, 4 of which were EBER-ISH positive cases. The distribution of IPI scores between the two groups is as depicted below.

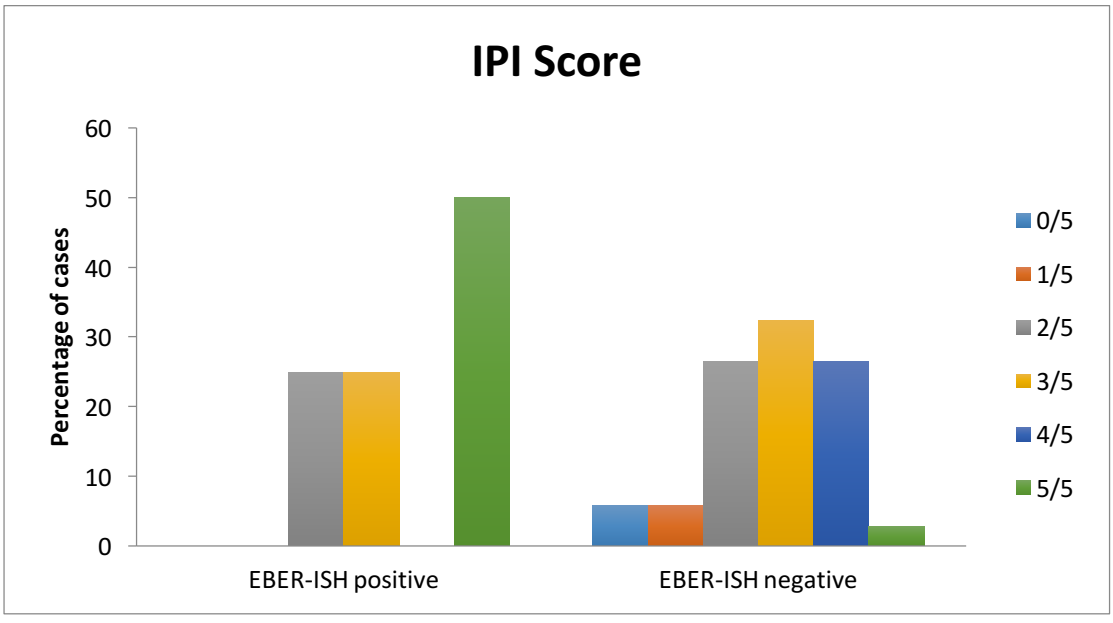


Figure 7: IPI score at presentation among EBER positive and EBER negative cases

Bone marrow involvement at presentation: Of the total 129 cases of DLBCL in patients older than 45 years of age in our study, 92 cases had undergone a bone marrow biopsy at presentation. This included 6 EBER-ISH positive cases and 86 EBER-ISH negative cases. 1 among the 6 EBER-ISH positive cases and 17 among the 86 EBER-ISH negative cases showed bone marrow involvement by lymphoma at presentation.

Histologic features**:**

Pattern of lymphoma infiltrate: The pattern of lymphoma infiltrate in lymph node biopsy sections was assessed based on the architecture at low power microscopic examination. This was broadly classified as diffuse and vaguely nodular. Cases that had a mixture of both patterns were further graded based on the relative proportion of the vaguely nodular pattern. 5 of the 7 (71.4%) cases of EBER-ISH positive DLBCL showed a diffuse pattern, while a mixed diffuse and nodular pattern and a vaguely nodular pattern were seen in 1 case each respectively.

Among the 102 EBER-ISH negative cases of DLBCL, 80 (78.4%) showed a diffuse pattern, 14 (13.7%) showed a vague nodular pattern and 6 (5.8%) showed a mixed diffuse and nodular pattern. 1 case in addition showed many scattered cells on biopsy, with no recognizable pattern. The pattern could not be recognized in 1 case.

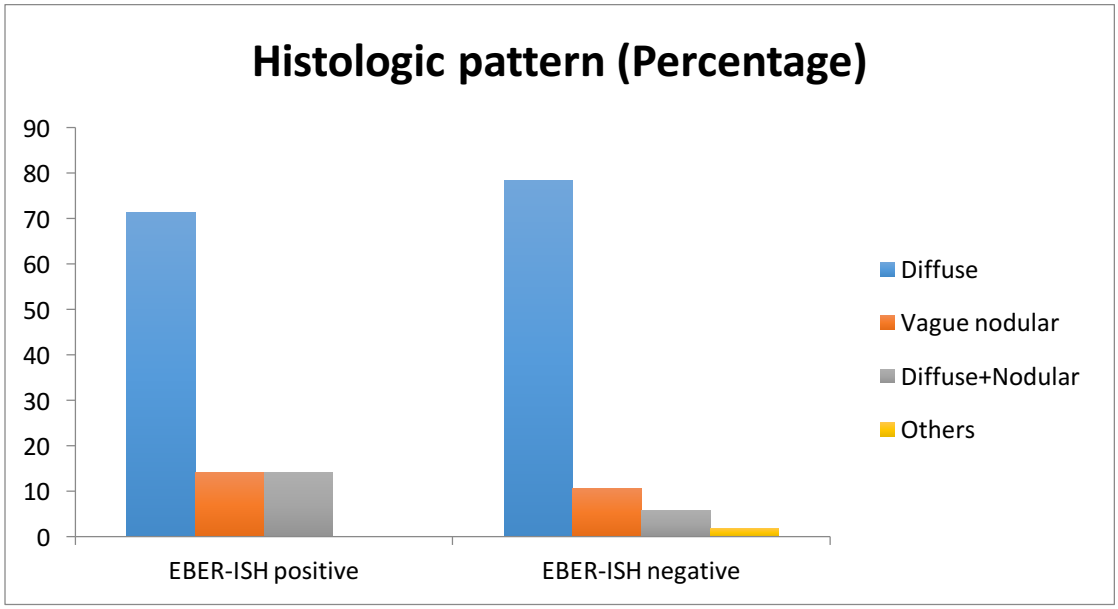


Figure 8: Pattern of tumour cell infiltrate among EBER-ISH positive and negative cases

Morphologic subtype: The cytologic subtypes of DLBCL that were identified in our study were centroblastic and anaplastic. The Immunoblastic pattern as defined by the WHO classification (>90% immunoblasts) was not seen. However, a subset of cases showed a predominantly centroblastic pattern but with increased proportion of immunoblasts not amounting to 90%. The other peculiar patterns observed included a centroblastic pattern with many clear cells and one with many spindled cells. The relative distribution of these patterns among EBER-ISH positive cases and EBER-ISH negative cases is depicted in the chart below.

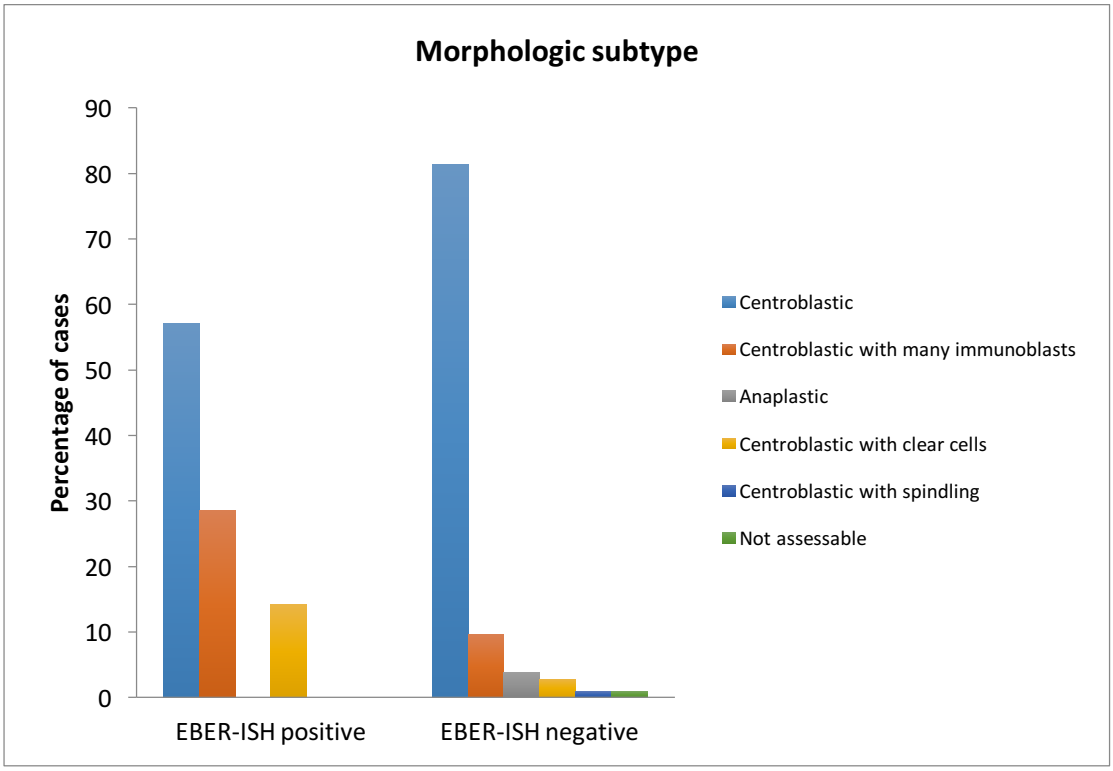


Figure 9: Morphologic/ Cytologic subtypes of DLBCL among EBER positive and EBER negative cases

Reactive background: A reactive background was seen in all 7 EBER-ISH positive cases of DLBCL, with one case showing a marked prominence. Among the EBER-ISH negative cases, 9 cases showed no reactive background, 40 cases showed a focal/ mild reactive background of which 1 case had a prominent eosinophilic infiltrate, and 52 cases had a readily evident reactive background, 2 of which showed marked prominence. The reactive background could not be assessed in one of the negative cases.

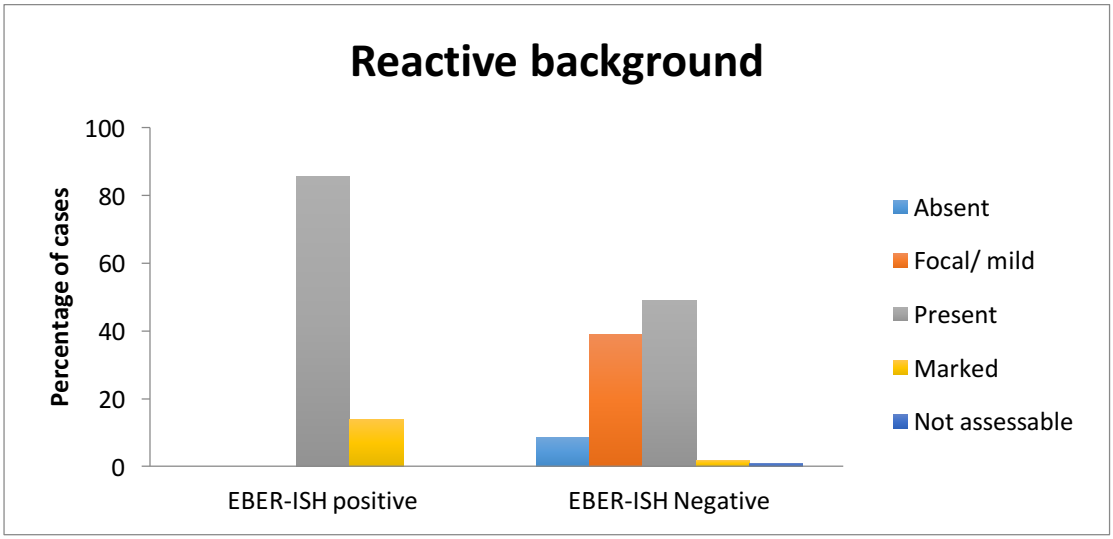


Figure 10: Degree of reactive background infiltrate among EBER-ISH positive and EBER-ISH negative cases

Necrosis: Necrosis was assessable in 6 EBER-ISH positive cases and 100 EBER-ISH negative cases. 2 of 6 (33.3%) positive cases showed frank necrosis. 20 of the 100 EBER-ISH negative cases showed frank necrosis and 28 EBER-ISH negative cases showed focal minimal or individual cell necrosis.

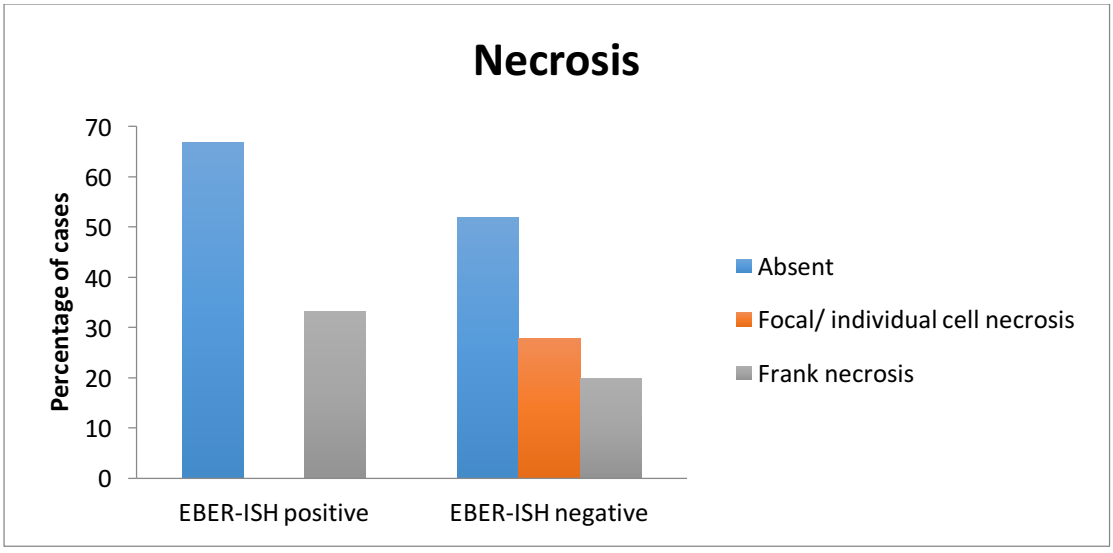


Figure 11: Degree of necrosis among EBER-ISH positive and negative cases of DLBCL

Tingible Body macrophages: Tingible body macrophages were evident in 4 of 7 (57.2%) and focally seen in 1 of 7 (14.2%) of EBER-ISH positive cases. In comparison, of the 102 EBER-ISH negative cases, 27 (26.5%) showed readily evident tingible body macrophages and 26 (25.6%) showed few/occasional tingible body macrophages. This difference was found to be statistically significant with a p value of 0.03.

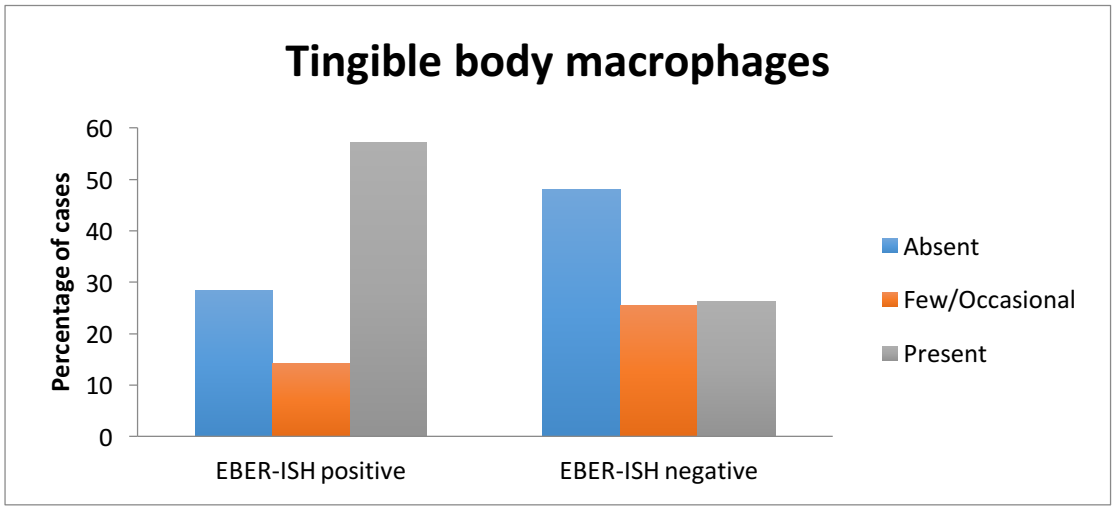


Figure 12: Proportion of tingible body macrophages in cases of EBER-ISH positive and negative cases of DLBCL

Multinucleate giant cells: Multinucleate giant cells were seen in 6 of 7 EBER-ISH positive cases, 1 of which showed a Hodgkin Reed Sternberg cell like morphology. Among the 102 EBER-ISH negative cases, multinucleate giant cells were seen in 47 cases, of which 12 showed a Hodgkin Reed Sternberg cell like morphology.

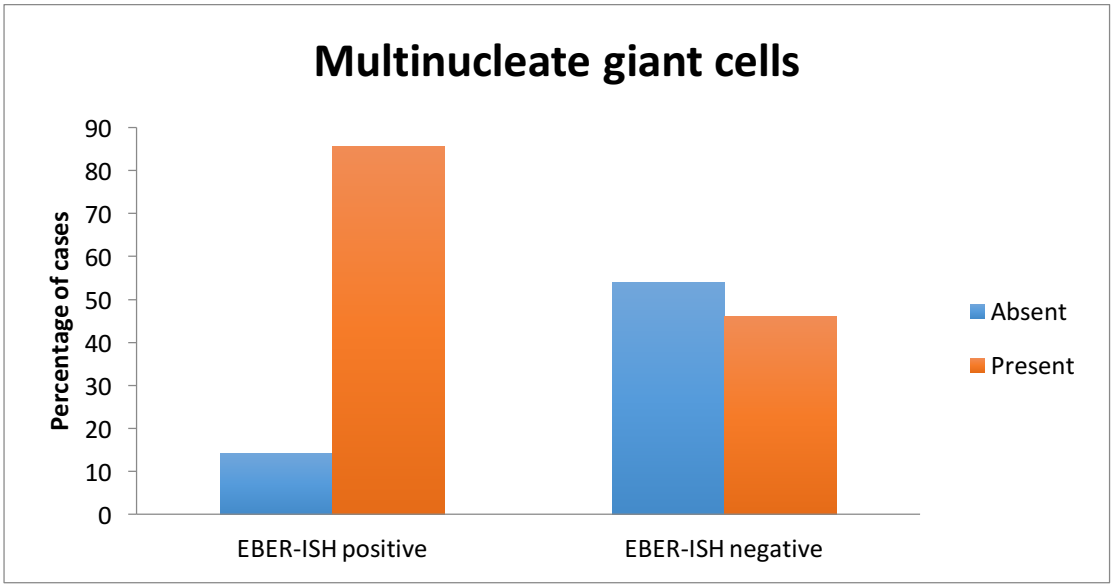


Figure 13: Presence of intratumoural multinucleate giant cells

Vascular proliferation: Vascular proliferation was present in all 7 cases of EBER-ISH positive DLBCL in our study. Among the 102 EBER-ISH negative cases, 77 cases showed prominent vascular proliferation and 13 cases showed only focal vascular proliferation. Vascular proliferation could not be assessed in 1 case. 1 case in addition showed prominent hyalinization of blood vessels.

This difference was found to be statistically significant with a p value of <0.01

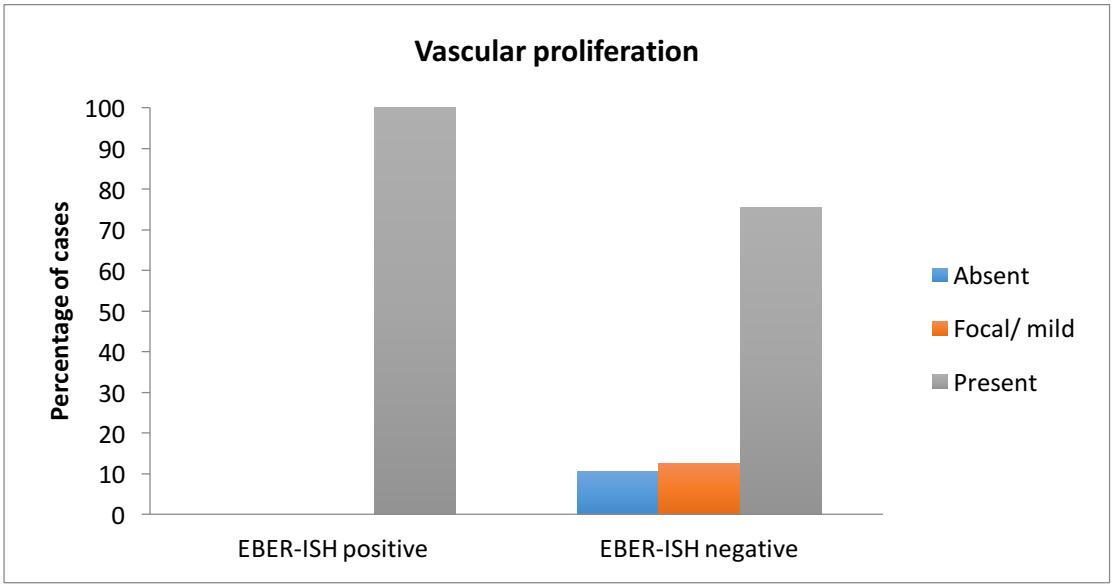


Figure 14: Intratumoural vascular proliferation in EBER-ISH positive and negative cases of DLBCL

Angioinvasion: Angioinvasion was seen only in 3 cases of DLBCL in our study, all of which were negative for EBER-ISH.

Fibrosis: Tumoural fibrosis was seen only focally in two cases of EBER-ISH positive DLBCL in this study. Among the 102 EBER-ISH negative cases, tumoural fibrosis was readily evident in 27 cases, and only focally seen in 24 cases. Fibrosis could not be assessed in 3 of the EBER-ISH negative cases.

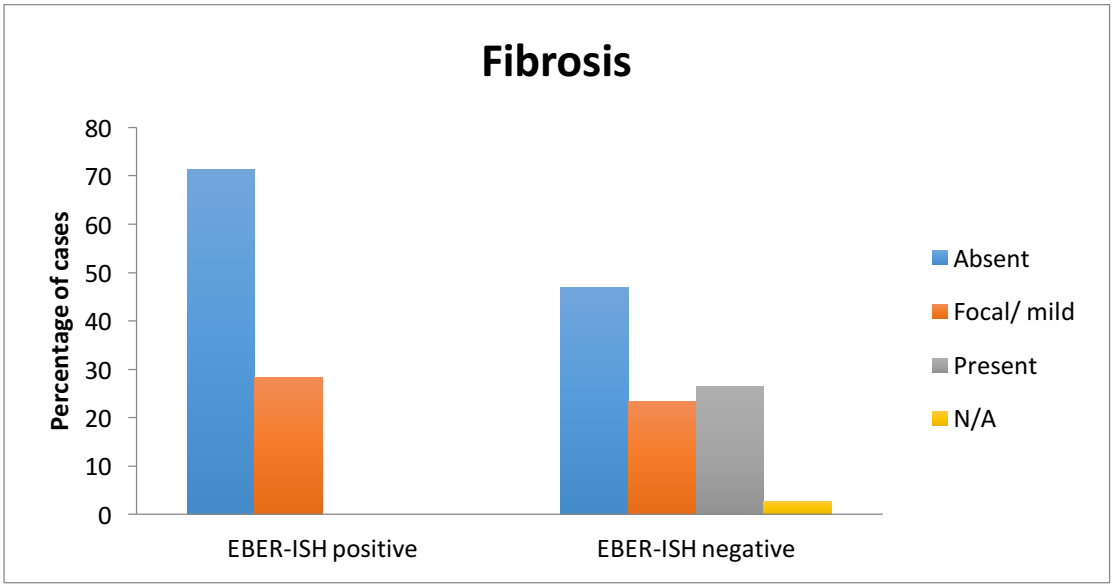


Figure 15: Intratumoural fibrosis among EBER-ISH positive and negative cases of DLBCL

Perinodal extension: Perinodal extension of tumour cells could be assessed in 4 of the 7 EBER-ISH positive cases and 80 of the 102 EBER-ISH negative cases. All 4 EBER-ISH positive cases and 76 of the remaining 80 EBER-ISH negative cases showed perinodal extension.

Table 3: Perinodal extension of tumour cell infiltrate in EBER-ISH positive and negative cases of DLBCL

	Present	Absent	Not assessable
EBER-ISH positive	4	0	3
EBER-ISH negative	76	4	22

Immunohistochemistry

CD3: slides for CD3 IHC were unavailable in 13 cases, including 3 which were EBER-ISH positive and 10 which were EBER-ISH negative. The remaining cases of EBER-ISH positive DLBCL showed <30% CD3 positive cells in 1 case, 30%-60% in 2 cases and >60% in 1 case. Among the remaining 92 cases of EBER-ISH negative cases, <30% CD3 positive cells were seen in 62 cases, 30% to 60% in 22 cases and >60% in 8 cases.

Table 4: Percentage of background CD3 positive T cells seen in EBER-ISH positive and negative cases of DLBCL

	<30%	30%-60%	>60%	N/A
EBER-ISH positive cases	1	2	1	3
EBER-ISH negative cases	62	22	8	10

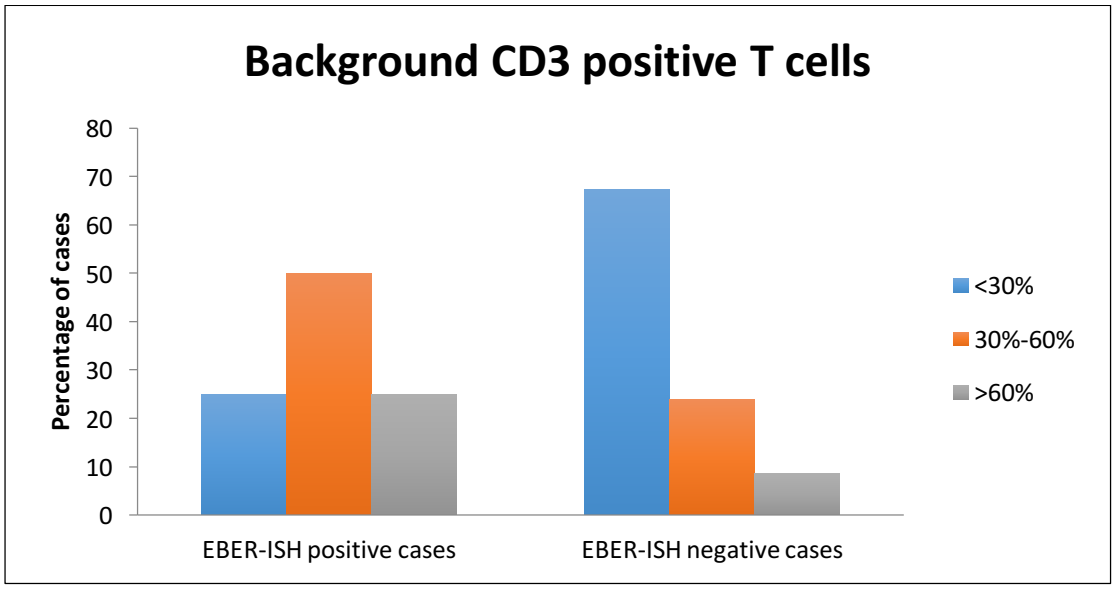


Figure 16: Percentage of cases with mild, moderate and marked background CD3 positive T cell infiltrate

CD20: The pattern of CD20 positive B cells closely reflected the morphologic patterns of tumour cell infiltrates seen on the initial Hematoxylin and Eosin stained slides.

MIB-1 proliferation index: The MIB-1 immunohistochemistry slides were unavailable in 2 cases including 1 each of EBER-ISH positive and negative cases. The average MIB-1 proliferation index in the EBER-ISH positive group was 84.2% (Range 75% - >95%) and that in the EBER-ISH negative group was 80.8% (Range 40% - >95%).

EBV-LMP1: EBV-LMP1 immunohistochemistry was done on 7 cases, including 3 EBER-ISH positive cases and 4 EBER-ISH negative cases. Among the EBER positives, one case was negative for EBV-LMP1 while the other two cases showed focal and prominent positivity respectively. The four cases, which were EBER-ISH negative, were also negative on EBV-LMP1 immunohistochemistry.

EBER-In Situ Hybridisation: EBER-ISH could be performed on 115 of the total 129 cases, of which 5 cases showed overwhelming background artifact and hence could not be assessed. 7 cases showed positivity for EBER-ISH, with one case containing 20-30% positive cells, 2 cases containing 50-60% positive cells, 1 case containing 60-70% positive cells and 3 cases with 80-90% positive cells. One case showed 5-10% positive cells, and this was considered as a negative case. Very occasional positive cells were seen in 8 cases, which were all considered as negative for

EBER-ISH. The positive cells could be easily identified at low power examination. The EBER-ISH positive cells showed a diffuse pattern in 3 cases, a nodular pattern in 2 cases and dispersed pattern in 2 cases.

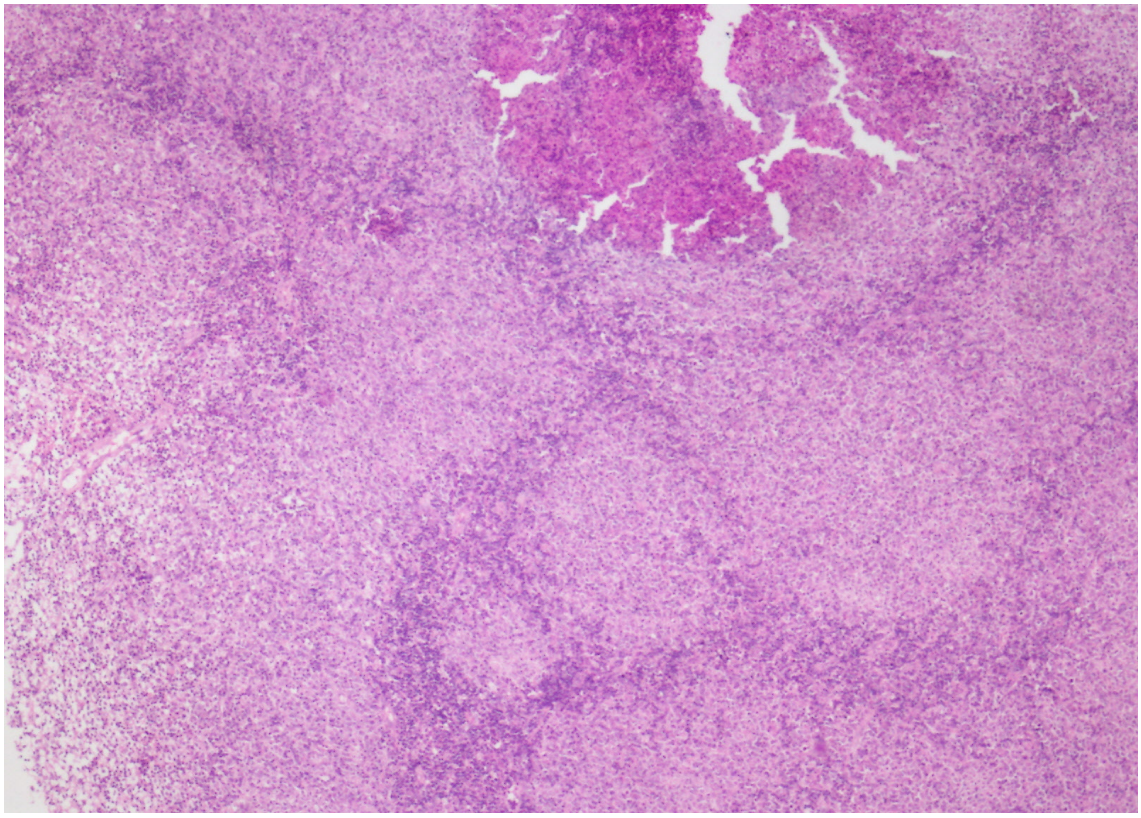
Table 5: Summary of EBER-ISH positive cases

Case no.	Percentage EBER-ISH positive	Pattern of EBER-ISH positive
1	50-60	Dispersed
2	80-90	Diffuse
3	80-90	Diffuse
4	80-90	Nodular
5	60-80	Diffuse
6	20-30	Dispersed
7	50-60	Nodular

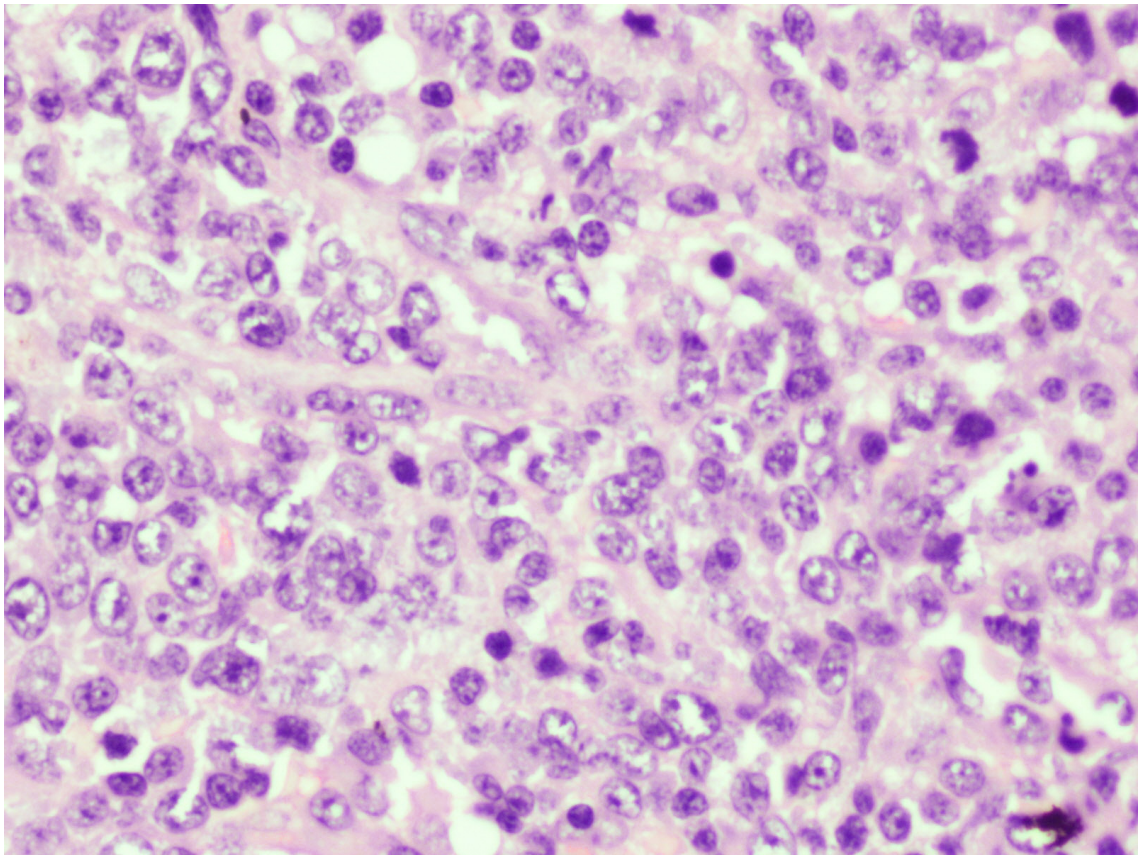
Table 6: Summary of all cases of Nodal DLBCL in patients >45 years of age and comparison with the EBER-ISH positive cases.

Histologic feature	Number (*Percentage*) of cases of all DLBCLs where present	Number (Percentage) of EBER-ISH positive DLBCLs where present
Pattern		
a. Diffuse	99 (*85*)	5 (*71.4*)
b. Nodular	5 (*4*)	1 (*14.3*)
c. Diffuse and vague nodular	13 (*11*)	1 (*14.3*)
Subtype		
a. Centroblastic	105 (*85*)	4 (*57.2*)
b. Anaplastic	4 (*3*)	-
c. Centroblastic with increased immunoblasts	10 (*8*)	2 (*28.6*)
d. Centroblastic with many clear cells	5 (*4*)	1 (*14.2*)
Reactive background		
a. Focal	56 (*44.8*)	
b. Readily evident	57 (*45.6*)	7 (*100*)
Necrosis		
a. Focal	38 (*29.5*)	
b. Readily evident	24 (*18.5*)	2 (*33*)
Tingible body macrophages		
a. Focal	37 (*28.9*)	
b. Readily evident	32 (*25*)	5 (*71.5*)
Multinucleate giant cells	56 (*45.2*)	6 (*85.7*)
Reed Sternberg like giant cells	15 (*11.6*)	1 (*14.3*)
Vascular proliferation		
a. Focal	25 (*19.5*)	
b. Readily evident	87 (*69*)	7 (*100*)
Angioinvasion	3 (*2.2*)	0
Fibrosis		
a. Focal	28 (*22*)	2 (*28.6*)
b. Readily evident	32 (*25.2*)	
Perinodal extension	87 (*69*)	4 (*100*)- [3 cases could not be assessed]

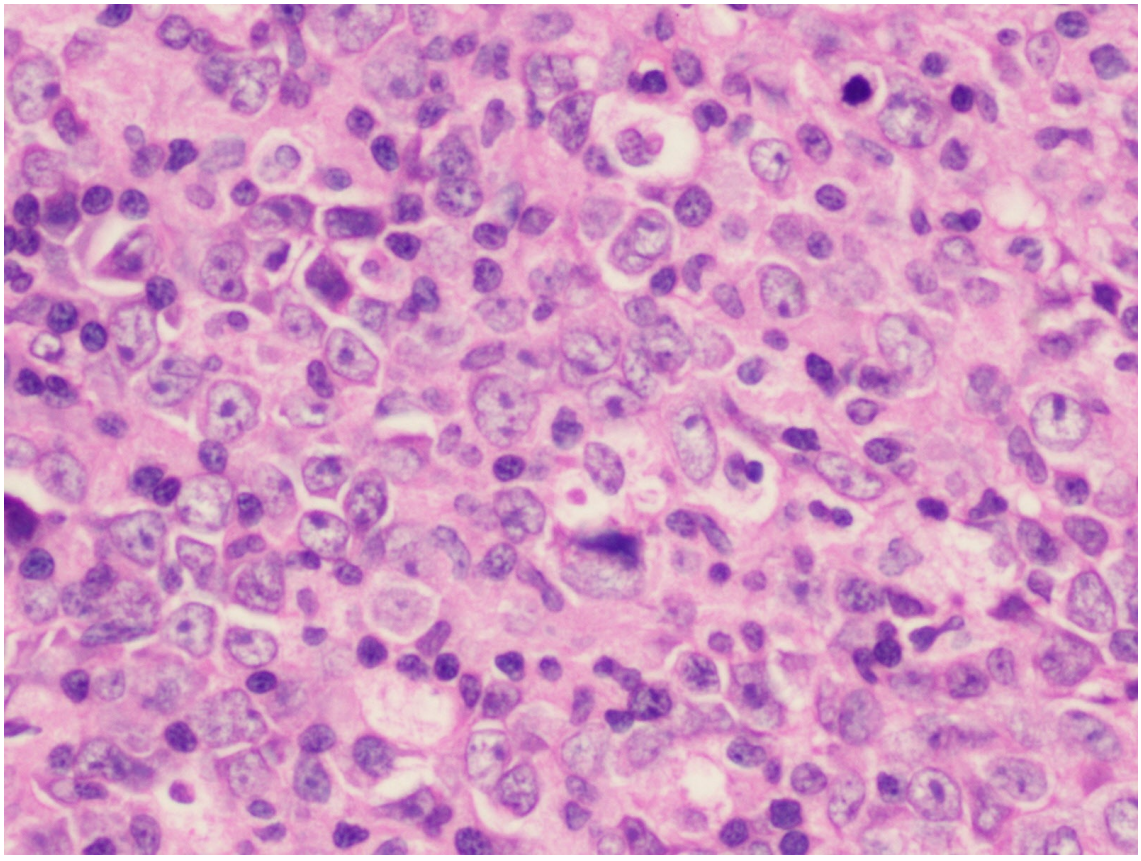
IMAGES



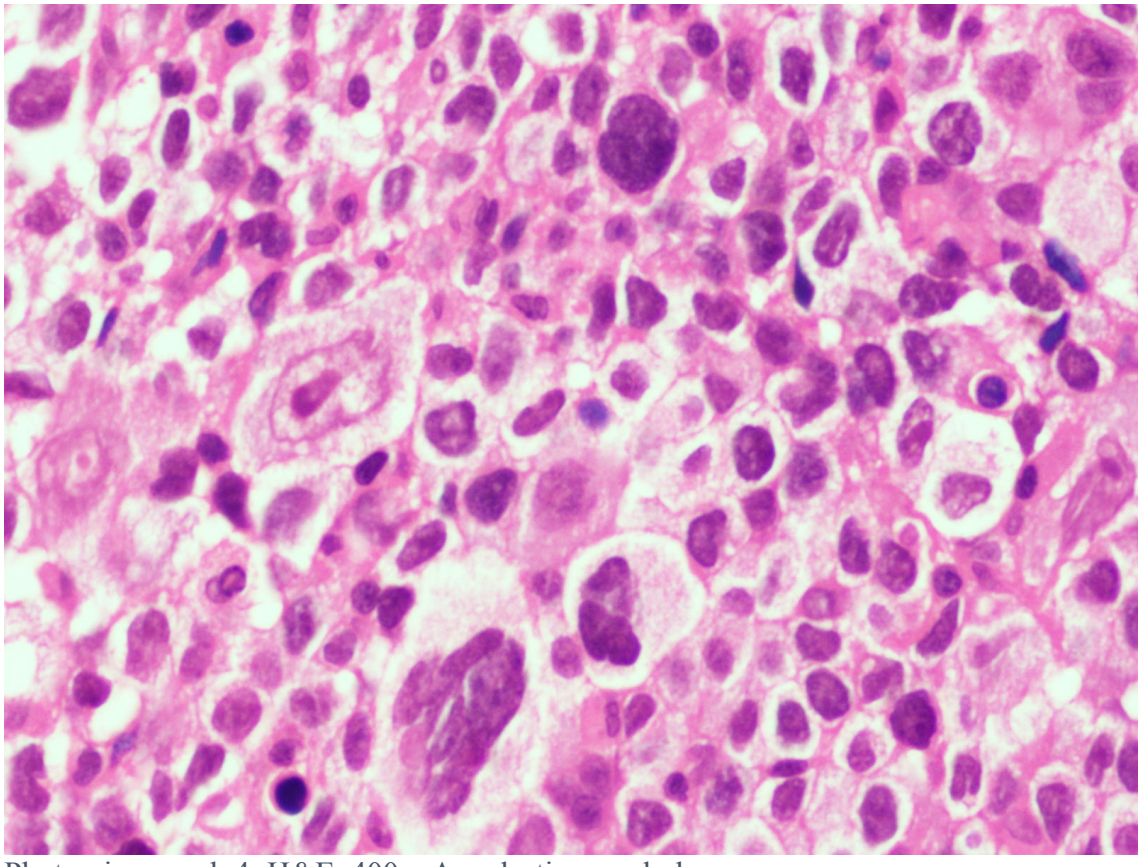
Photomicrograph 1: H&E, 40X- Vague nodular pattern



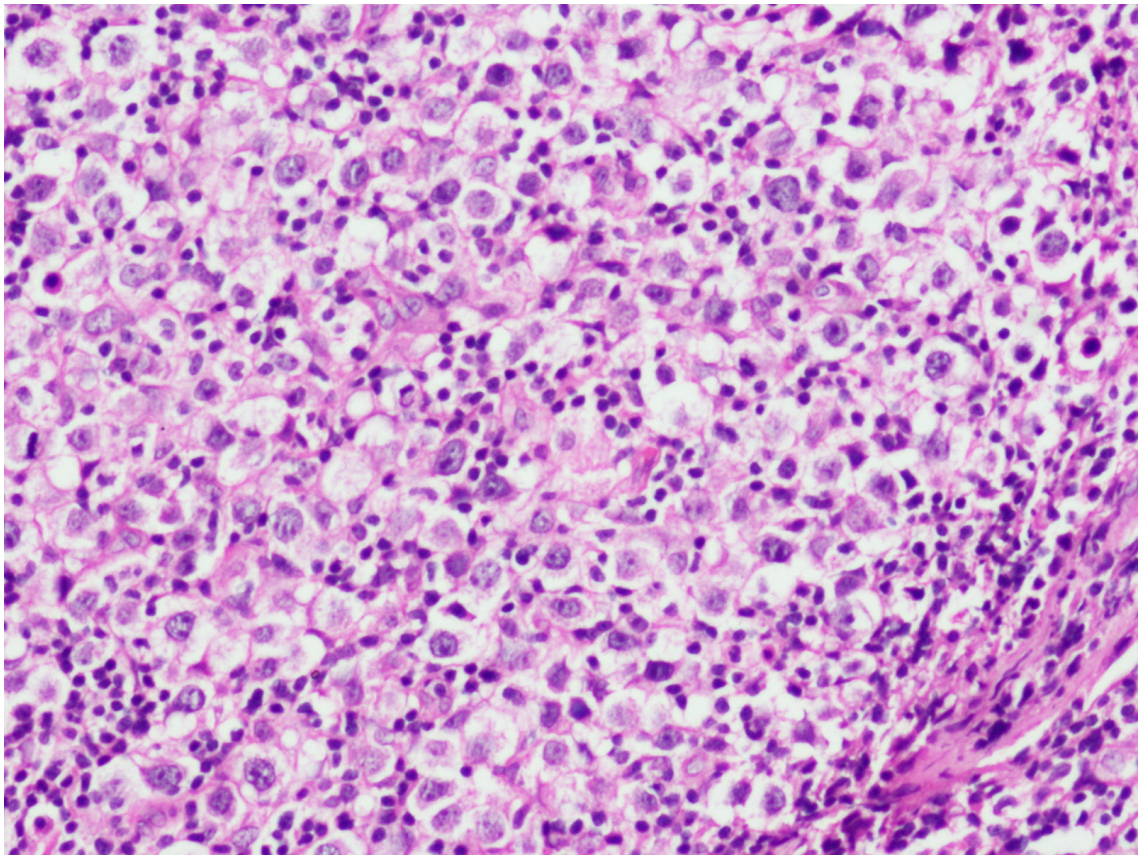
Photomicrograph 2: H&E, 400x, Centroblastic morphology



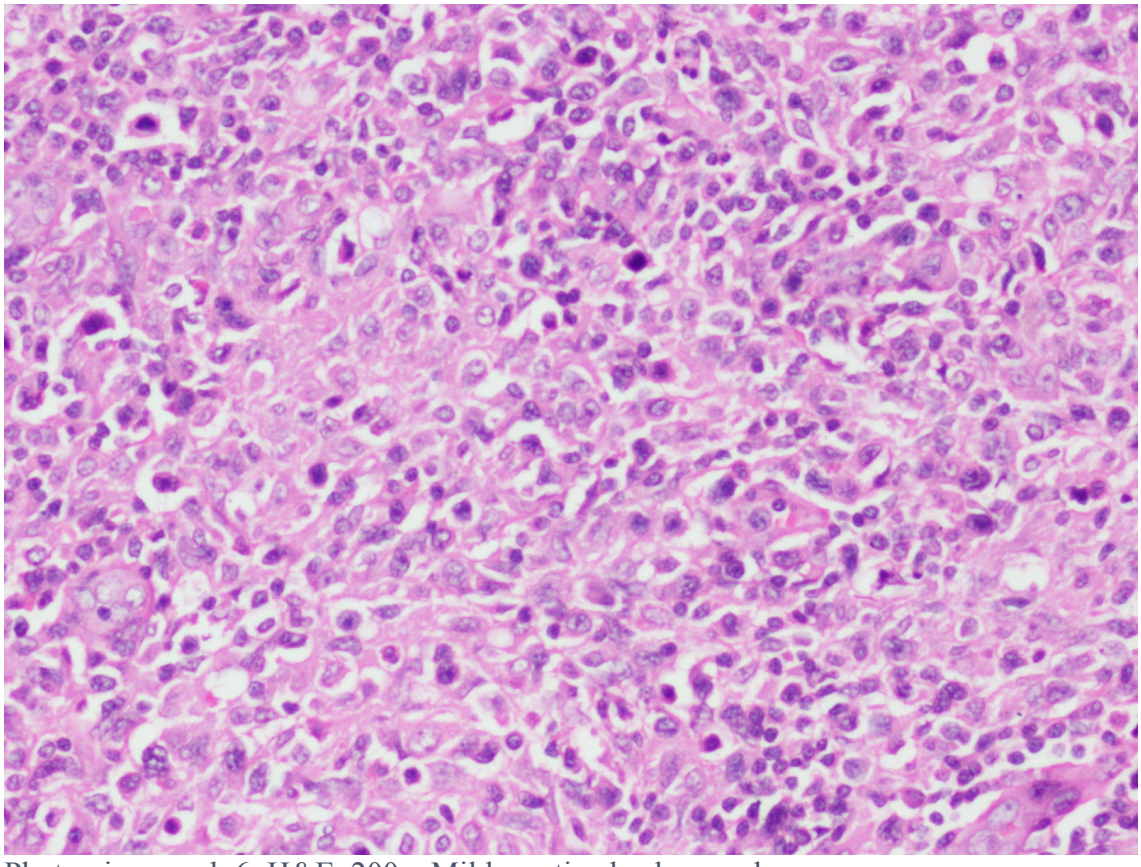
Photomicrograph 3: H&E, 400x, Centroblasts with many immunoblasts



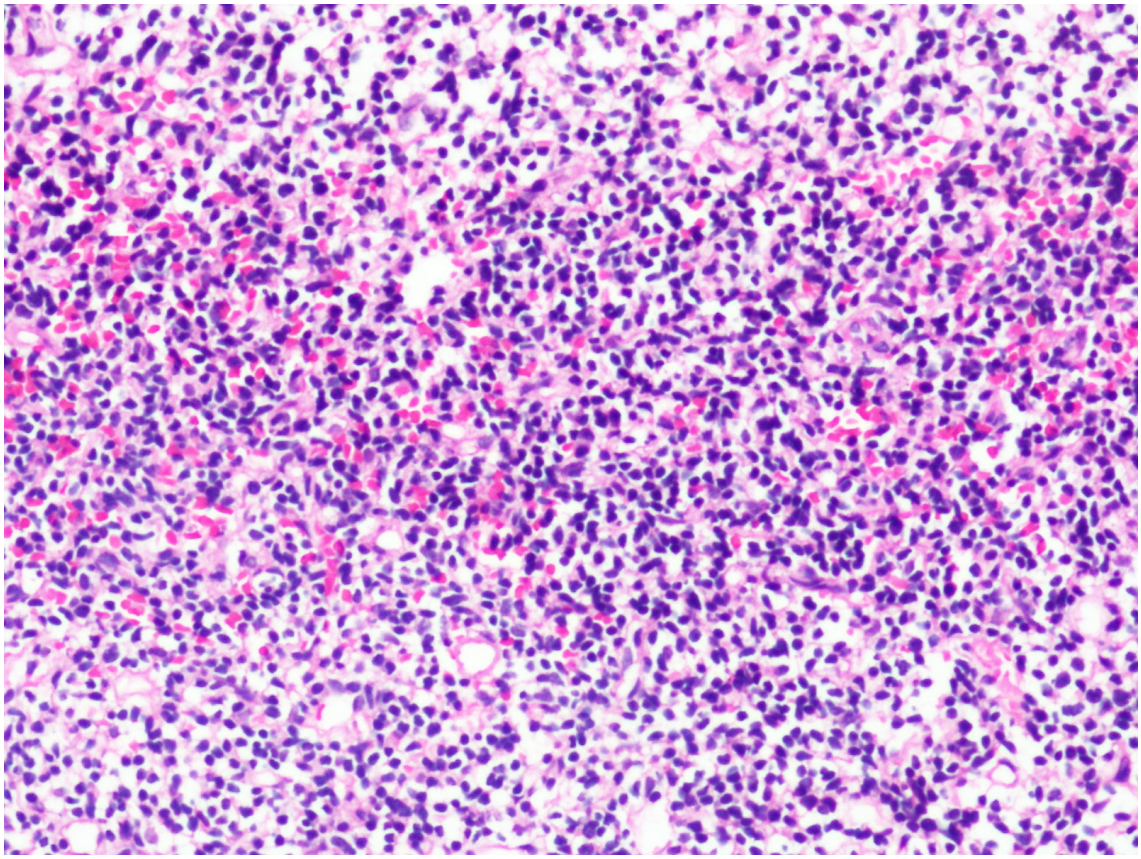
Photomicrograph 4: H&E, 400x, Anaplastic morphology



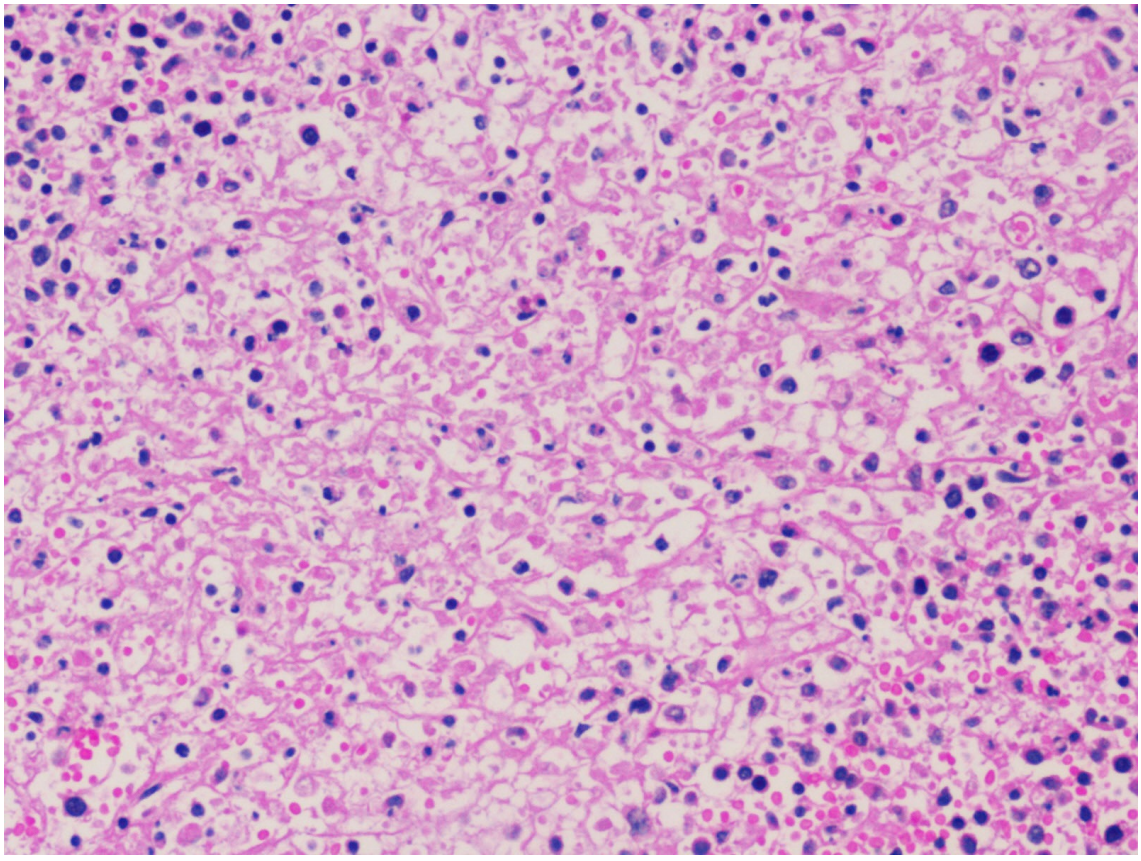
Photomicrograph 5: H&E, 200x, Predominance of clear cells



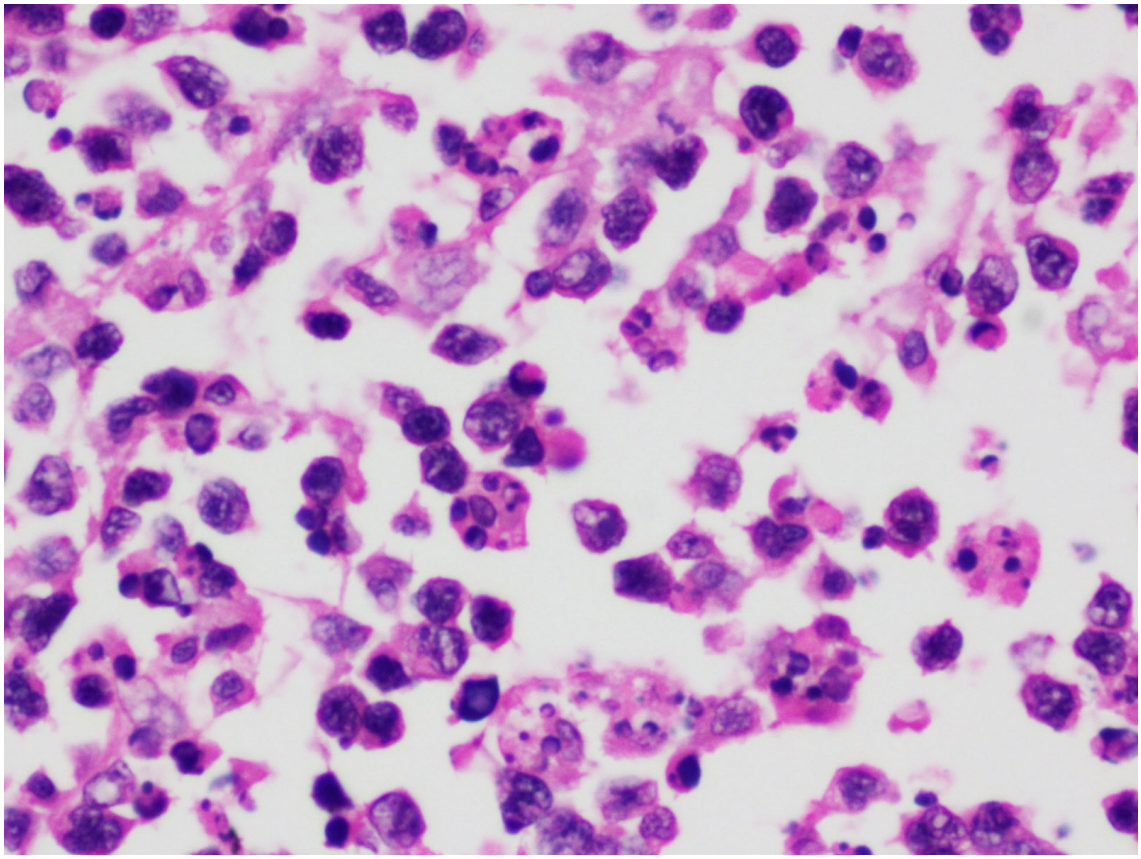
Photomicrograph 6: H&E, 200x, Mild reactive background



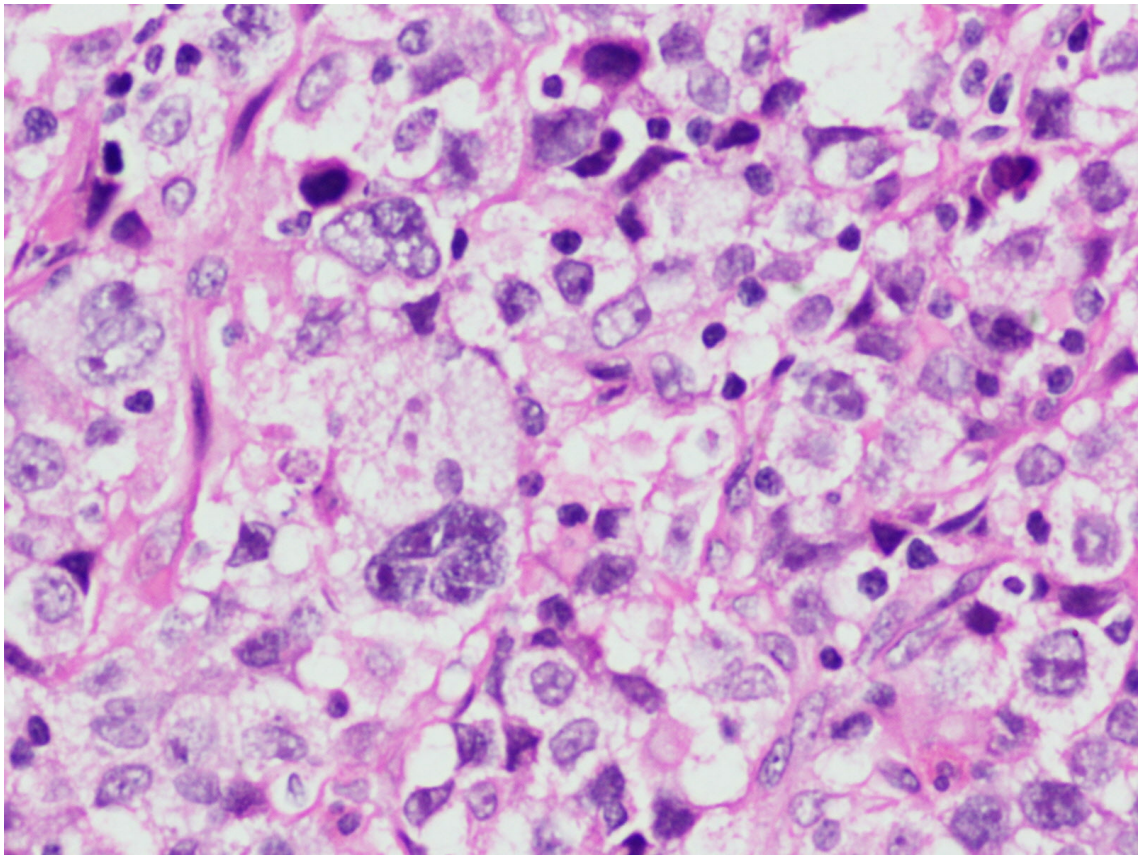
Photomicrograph 7: H&E, 100x, Marked reactive background



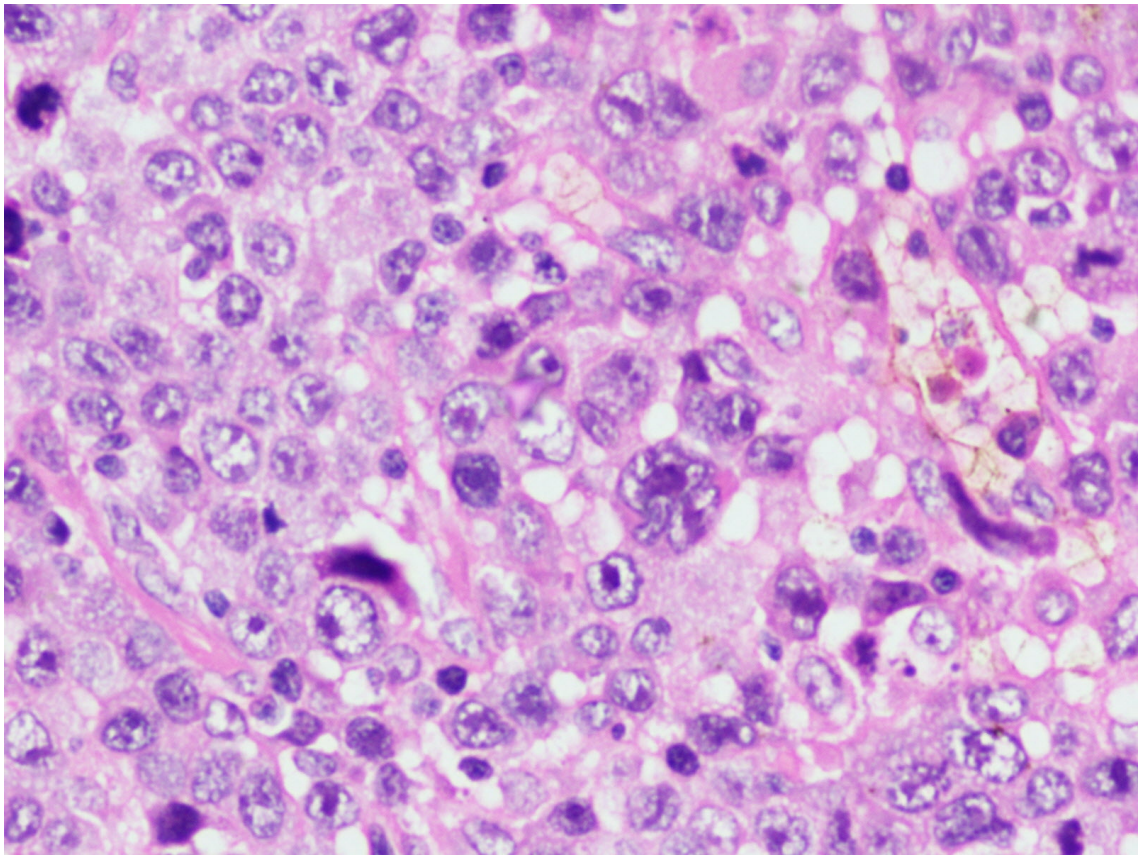
Photomicrograph 8: H&E, 100x, Necrosis



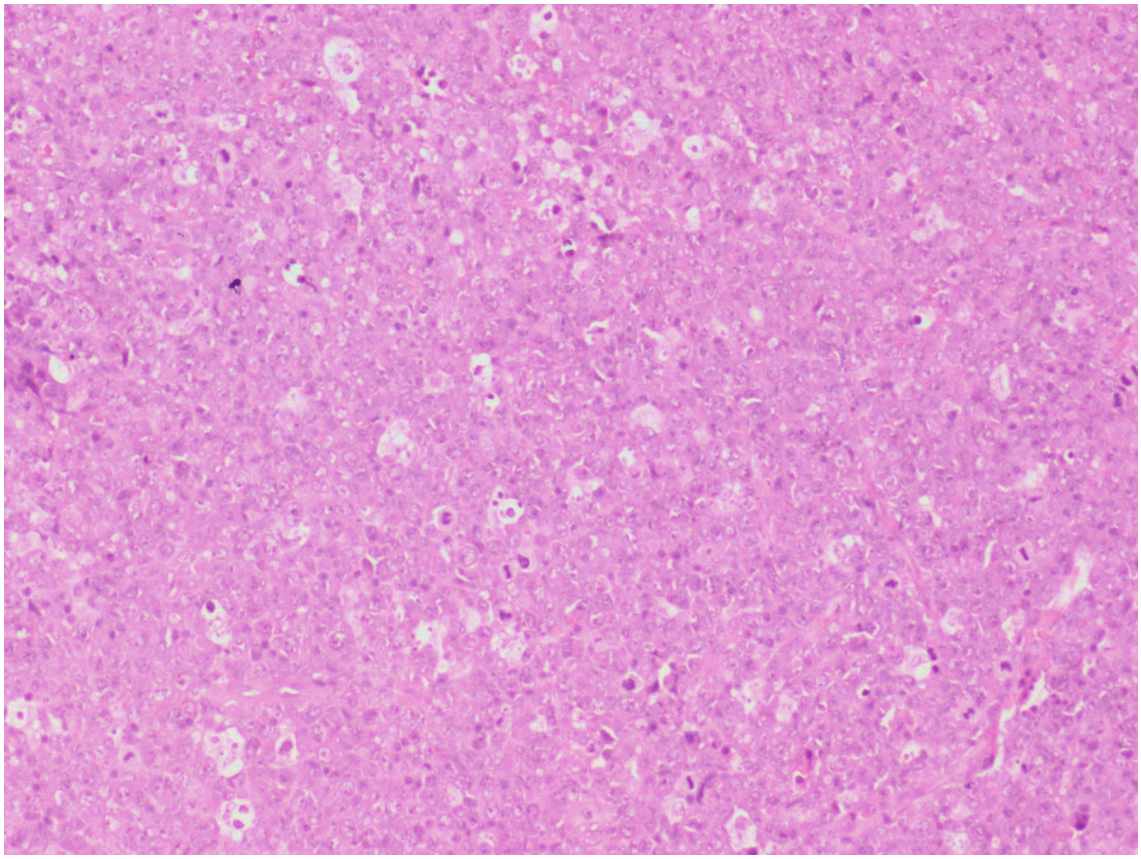
Photomicrograph 9: H&E, 400x, Apoptosis



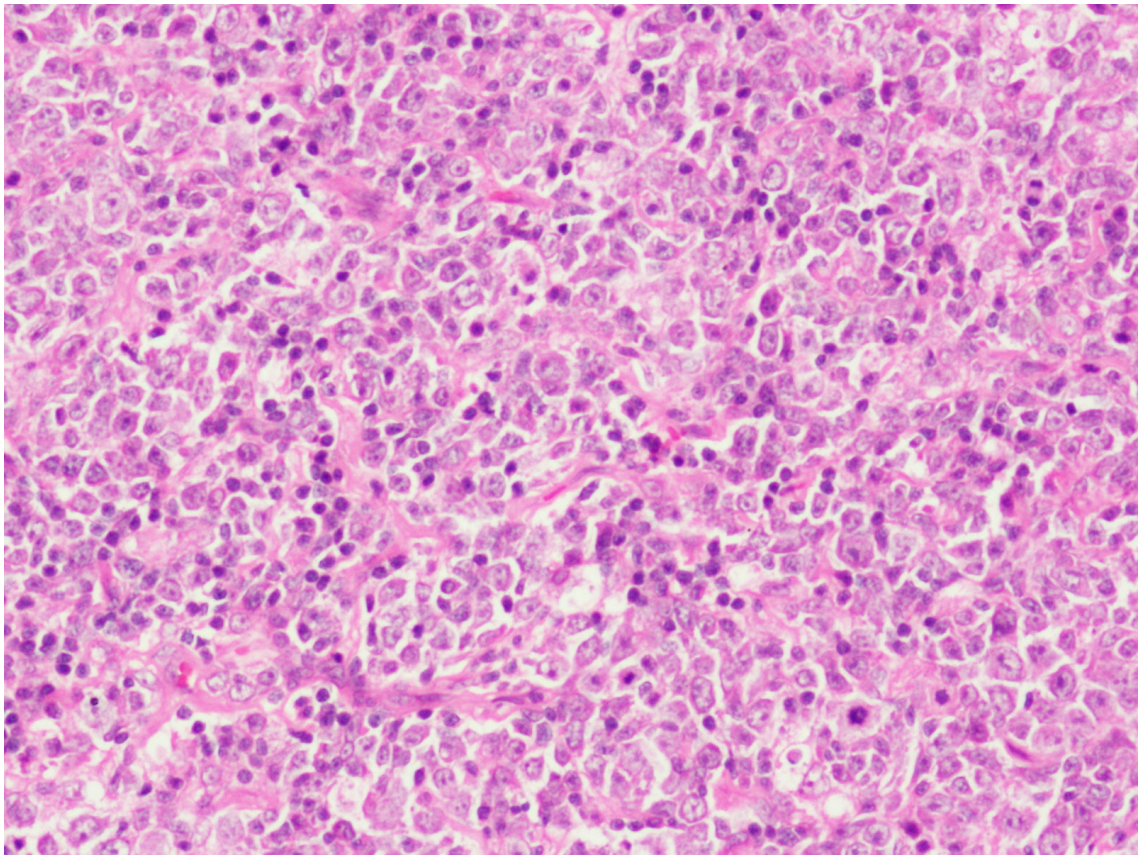
Photomicrograph 10: H&E, 400x, Multinucleate giant cells



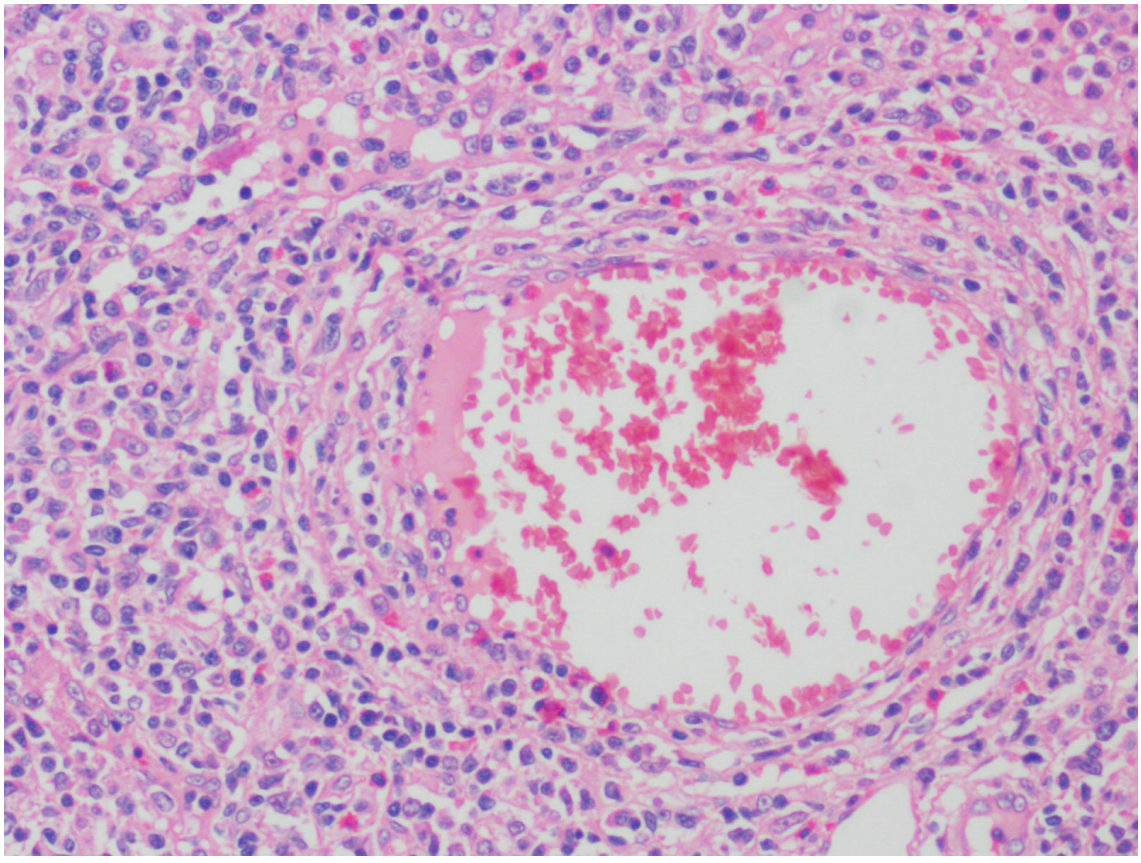
Photomicrograph 11: H&E, 400x, Reed-Sternberg like giant cell



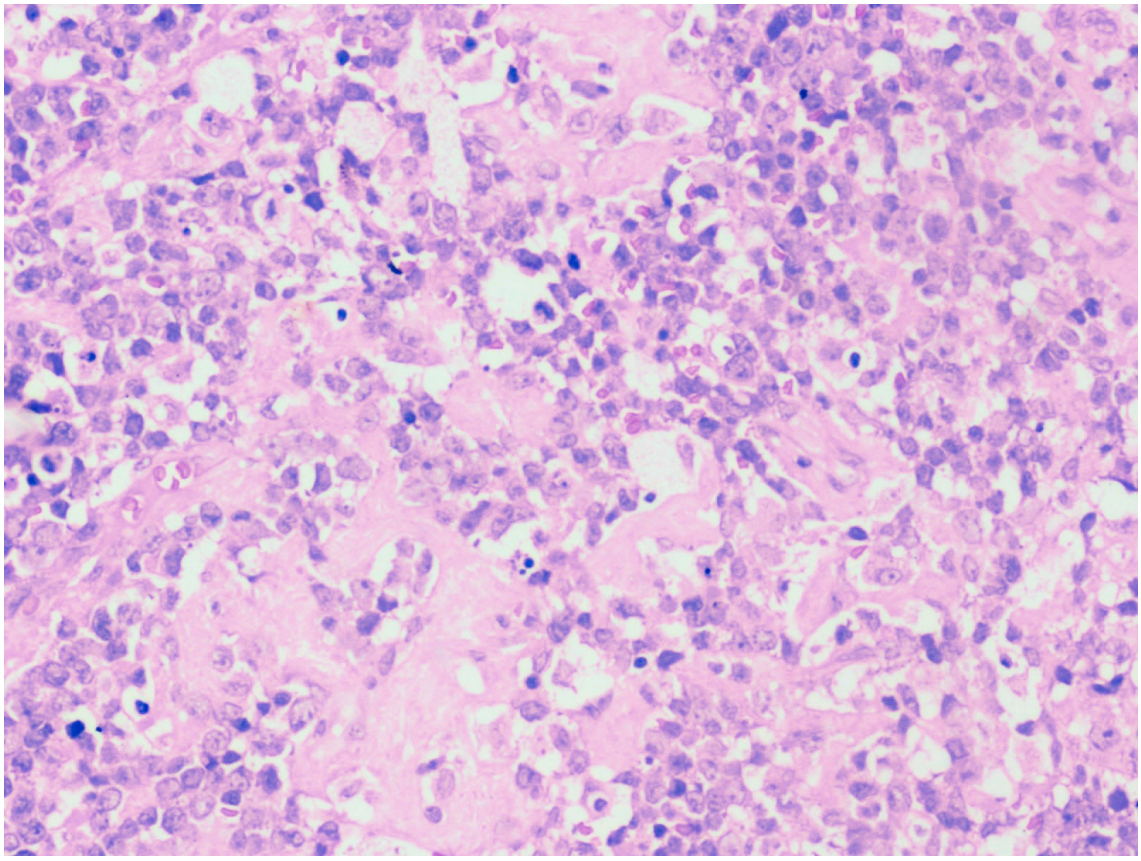
Photomicrograph 12: H&E, 100x, Tingible body macrophages



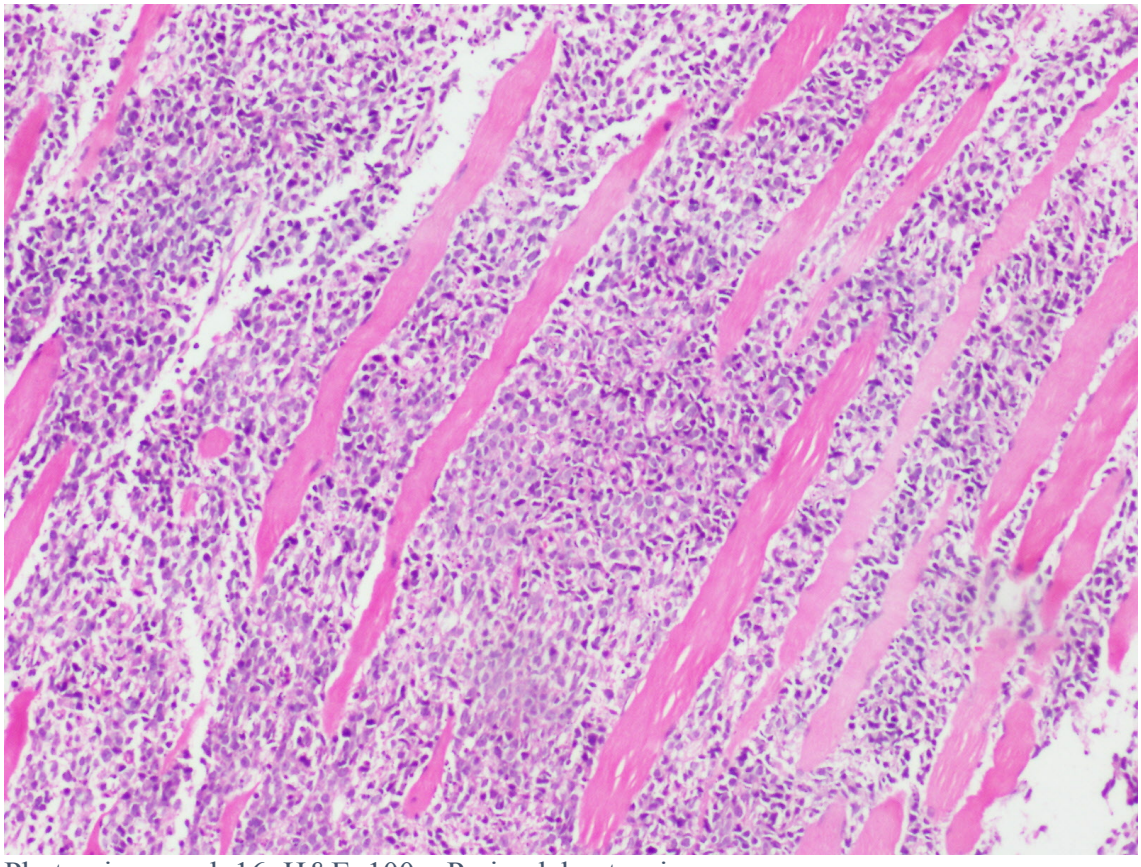
Photomicrograph 13: H&E, 200x, Vascular proliferation



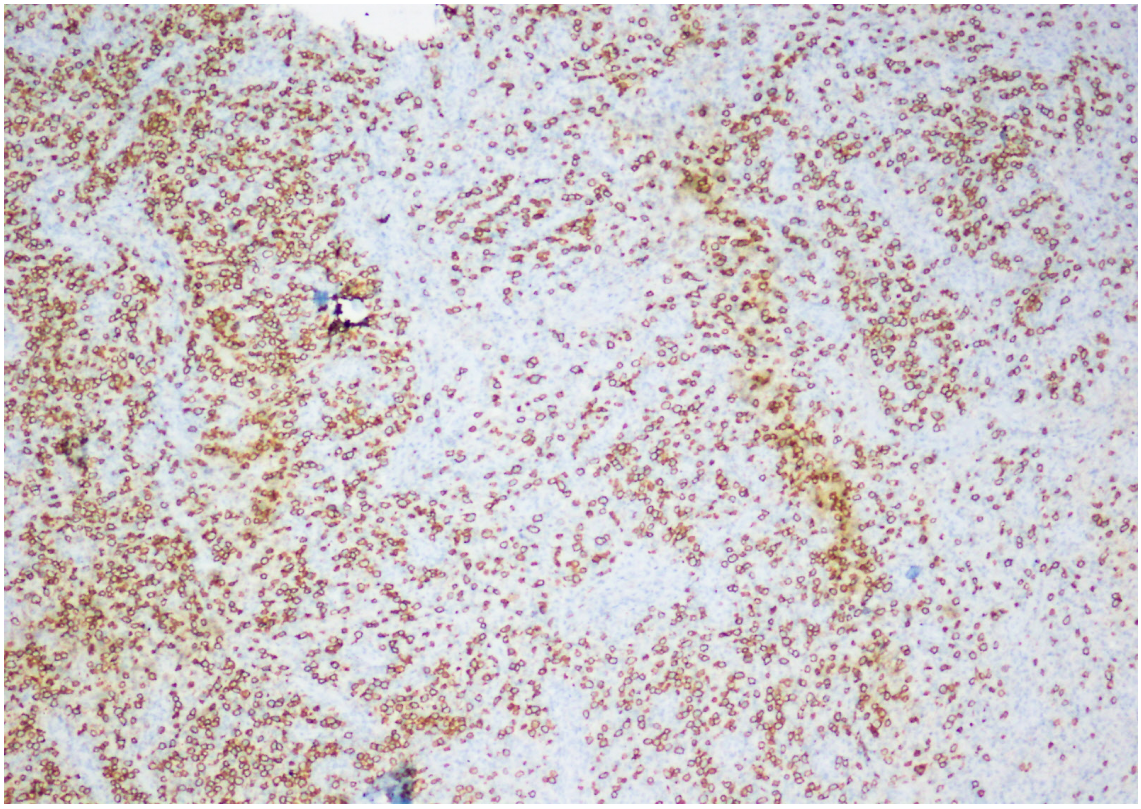
Photomicrograph 14: H&E, 200x, Angioinvasion



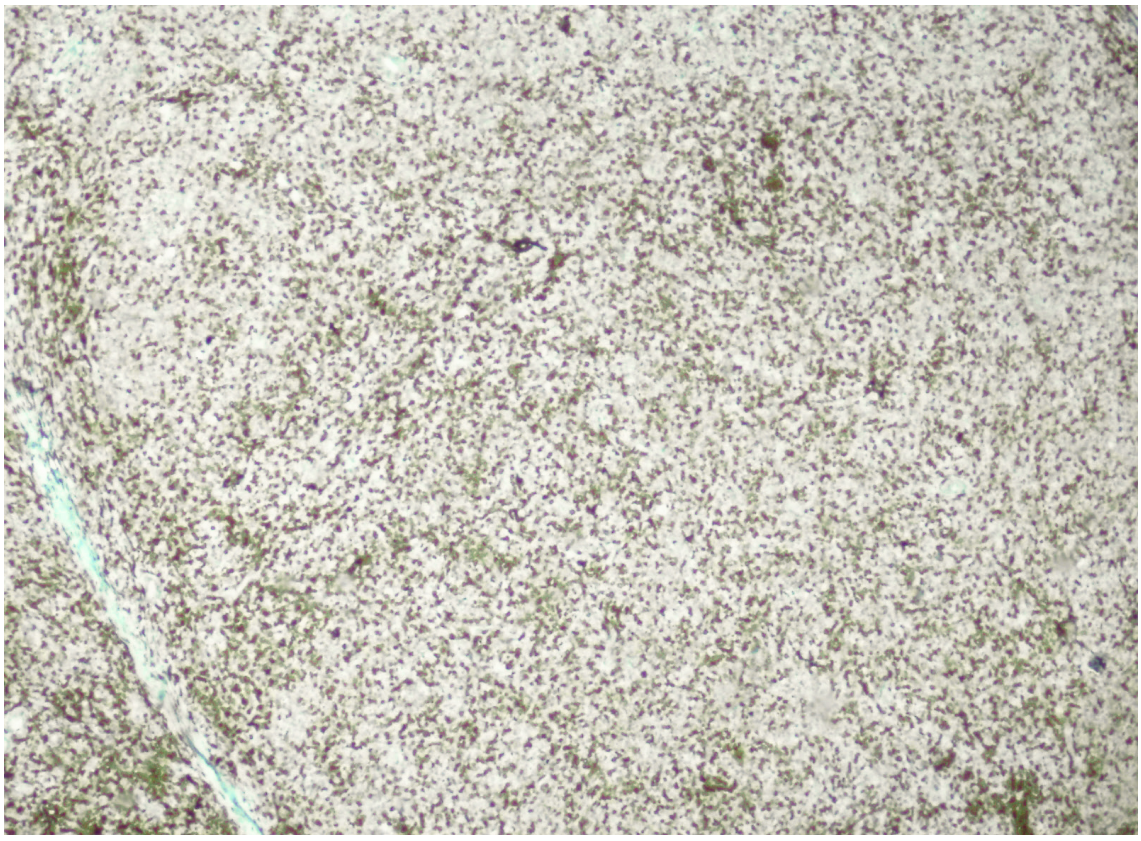
Photomicrograph 15: H&E, 200x, Intranodal fibrosis



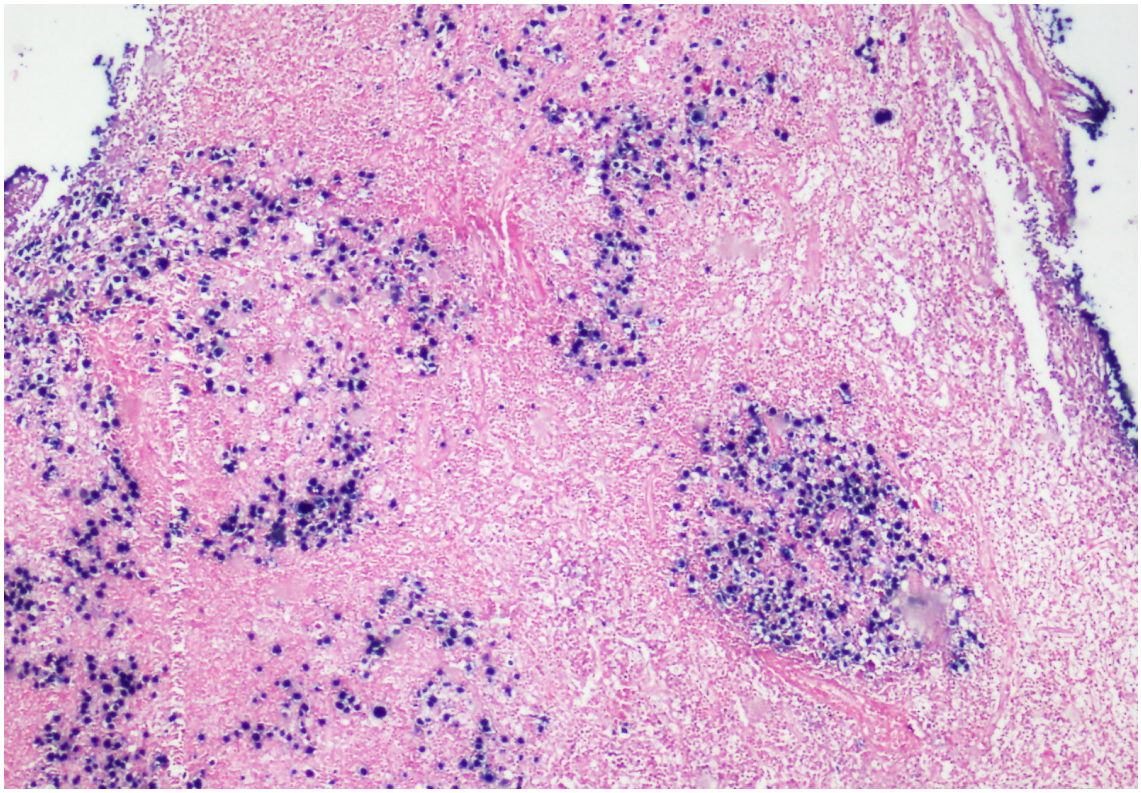
Photomicrograph 16: H&E, 100x, Perinodal extension



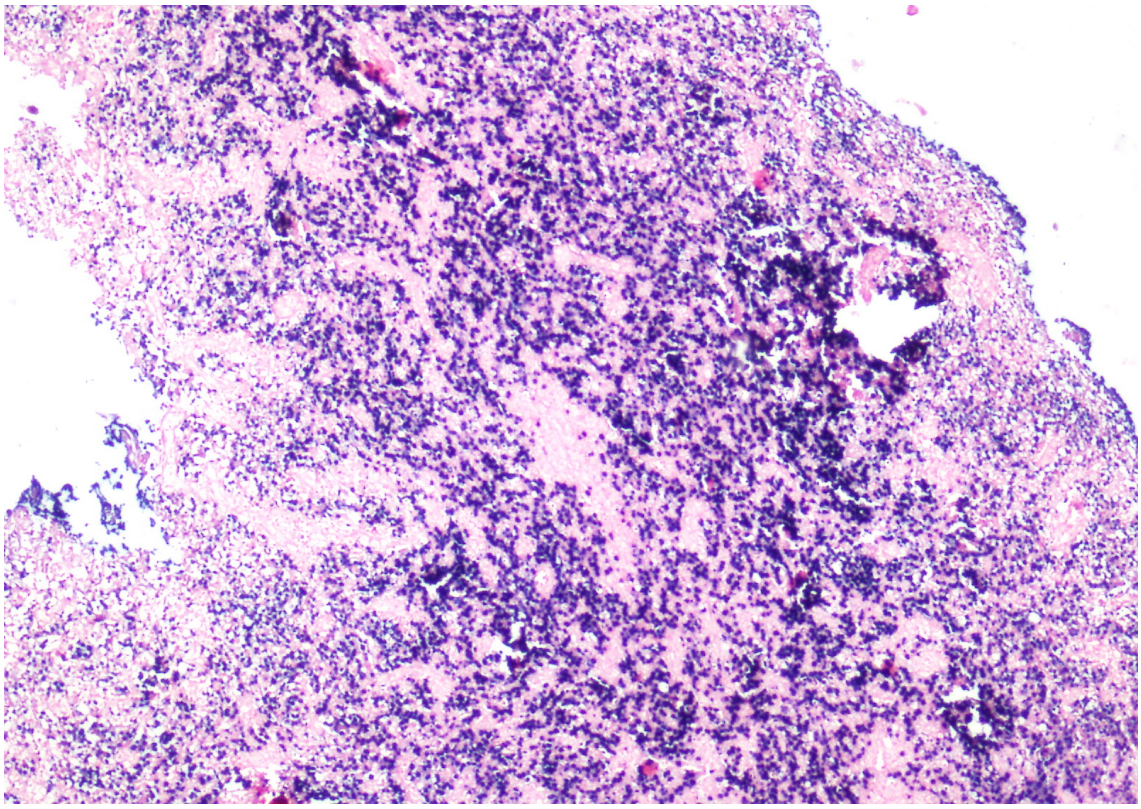
Photomicrograph 17: 100X- Scattered CD20 positive cells in 1 case



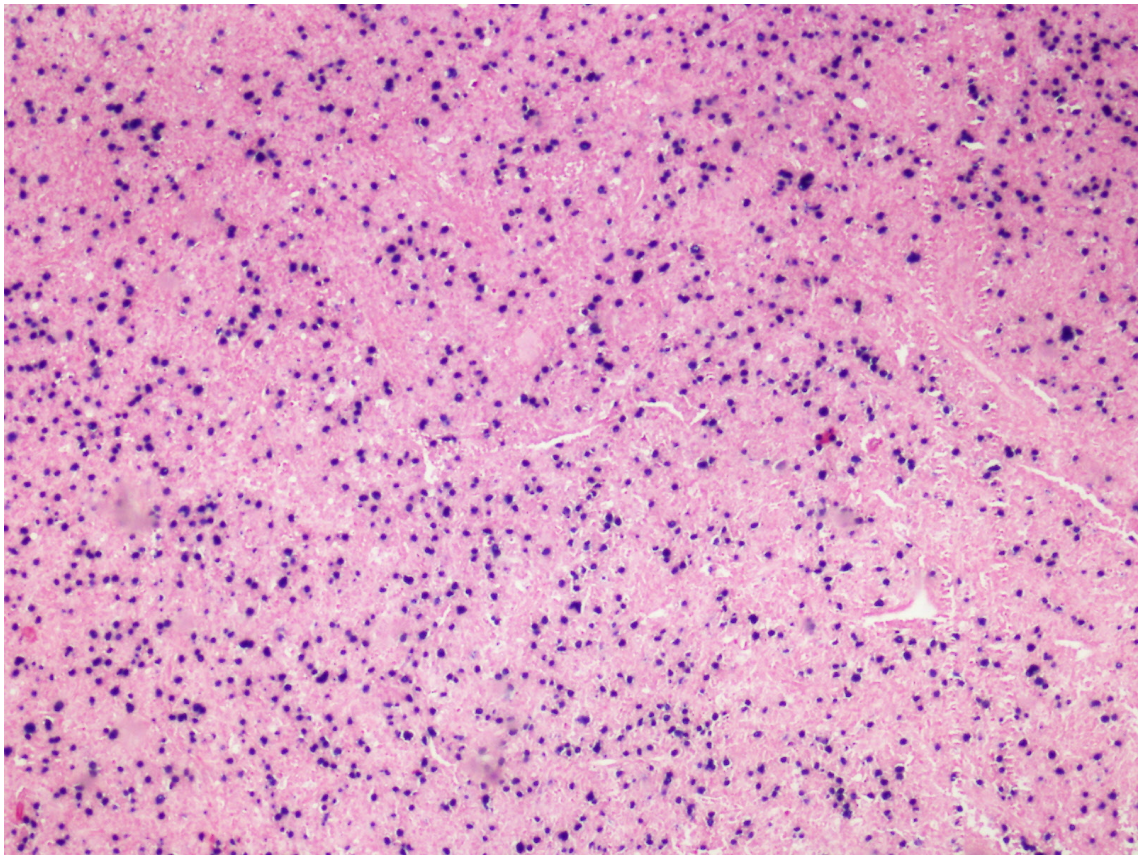
Photomicrograph 18: 100X- >60% CD3 positive T cells in a case with a marked reactive background



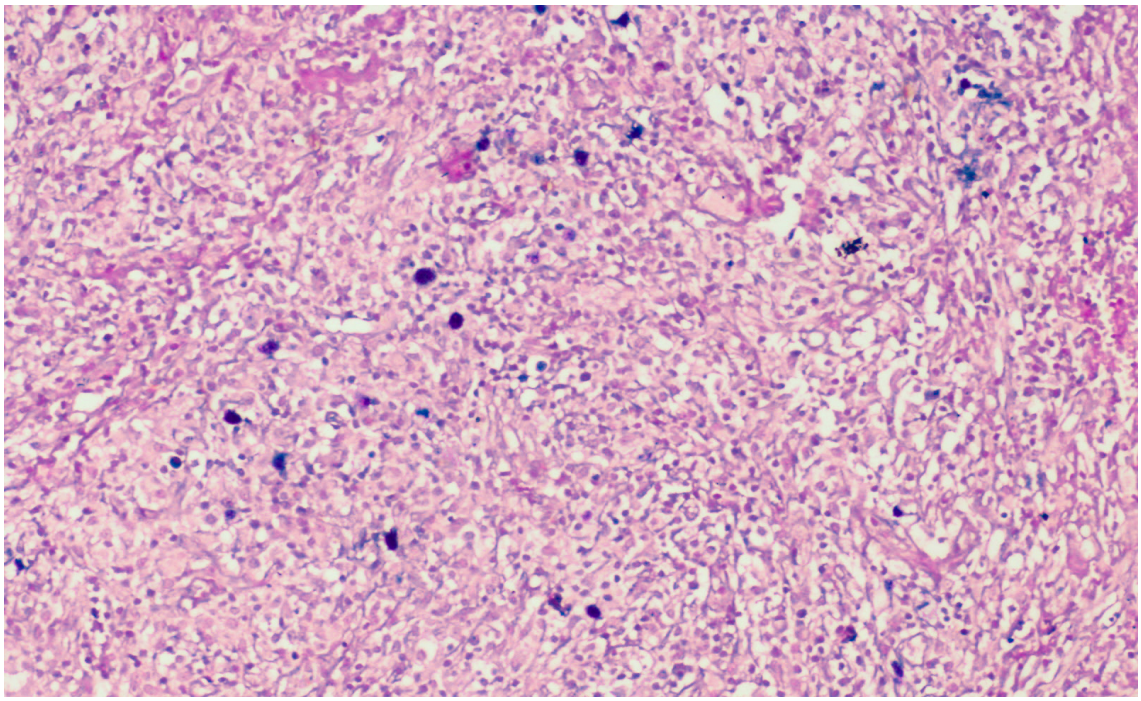
Photomicrograph 19: EBER-ISH, 40X-Nodular pattern of EBER-ISH positive cells



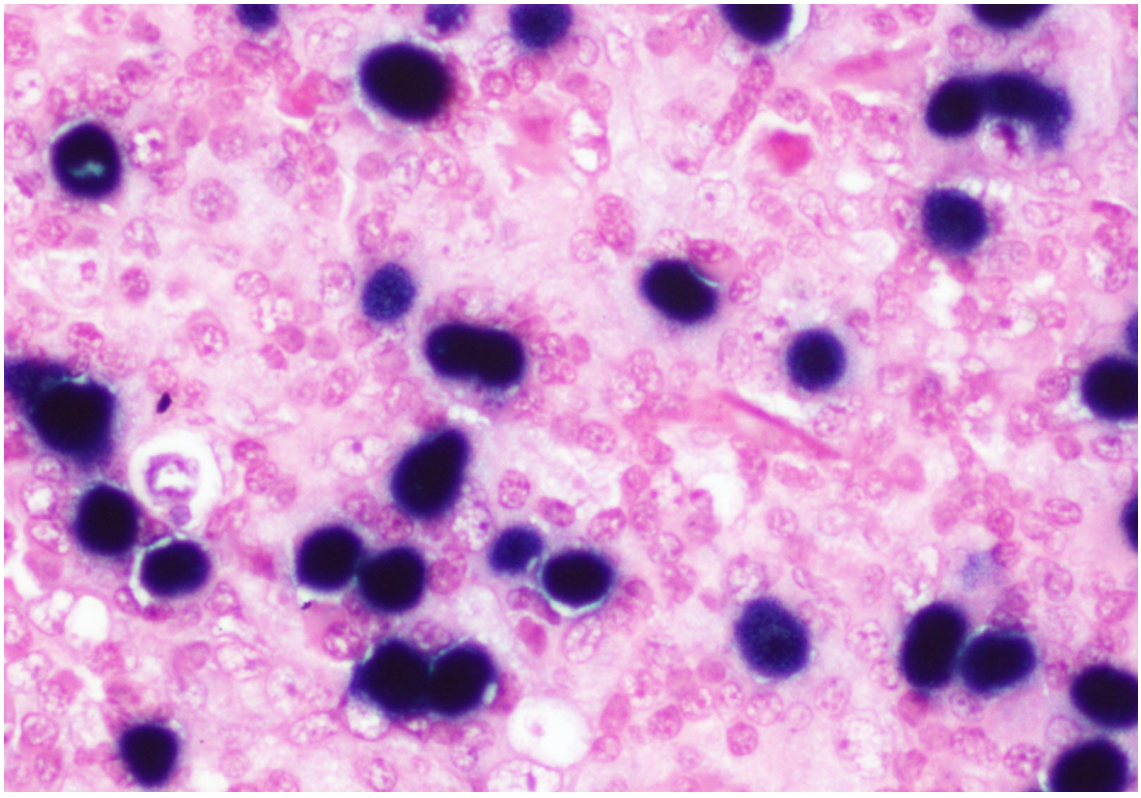
Photomicrograph 20: EBER-ISH, 40X-Diffuse pattern of positive cells



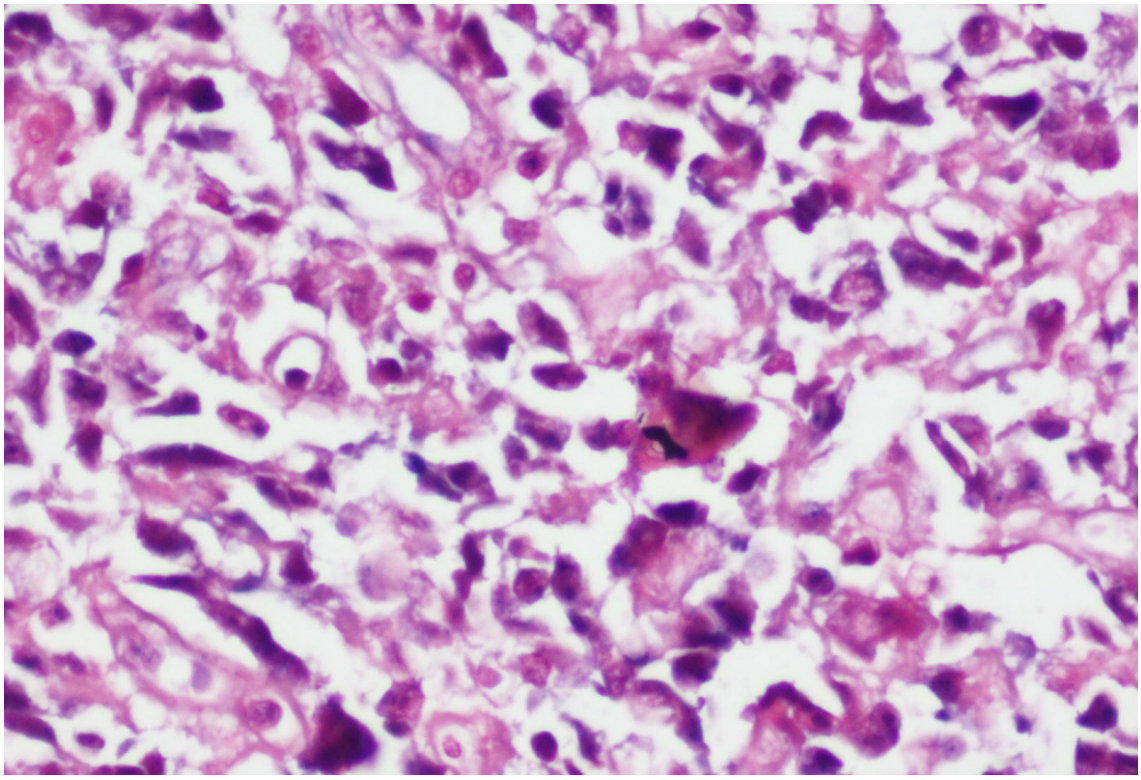
Photomicrograph 21: EBER-ISH, 40X-Scattered EBER-ISH positive cells (50-60%) in 1 case



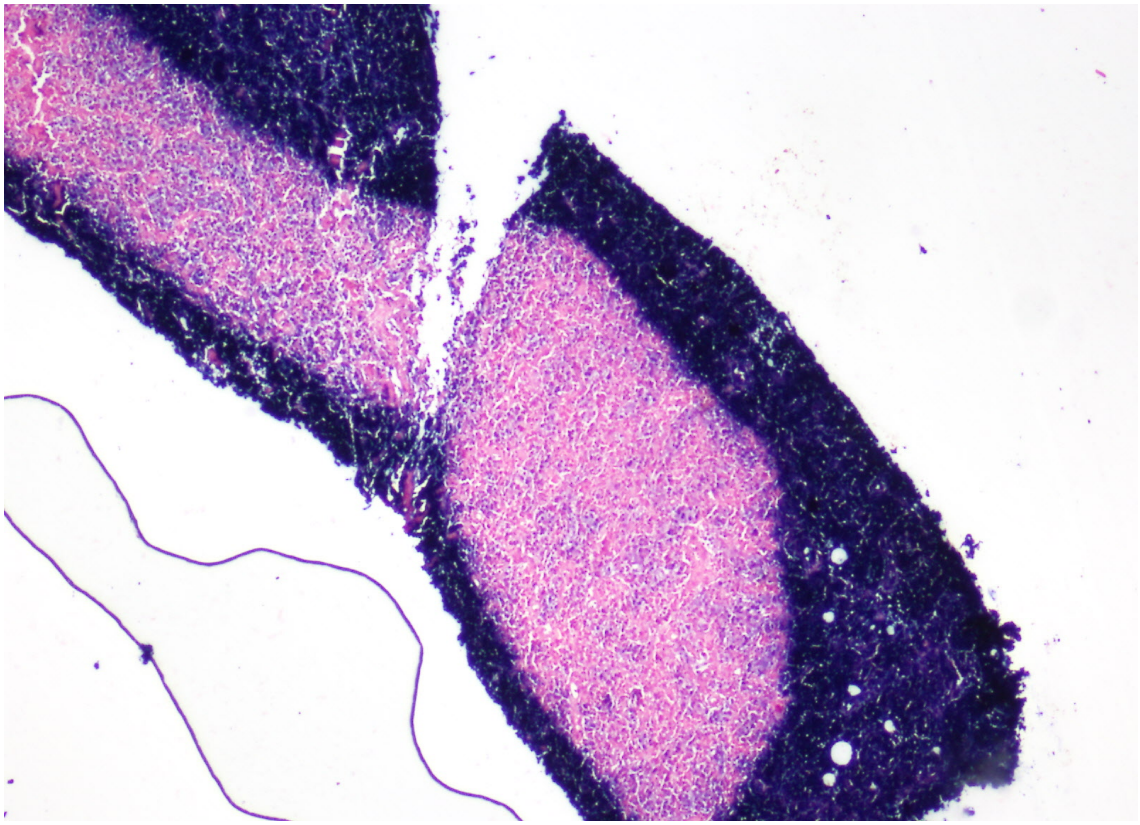
Photomicrograph 22: EBER-ISH, 100X-5% to 10% EBER-ISH positive tumour cells in 1 case, considered as negative



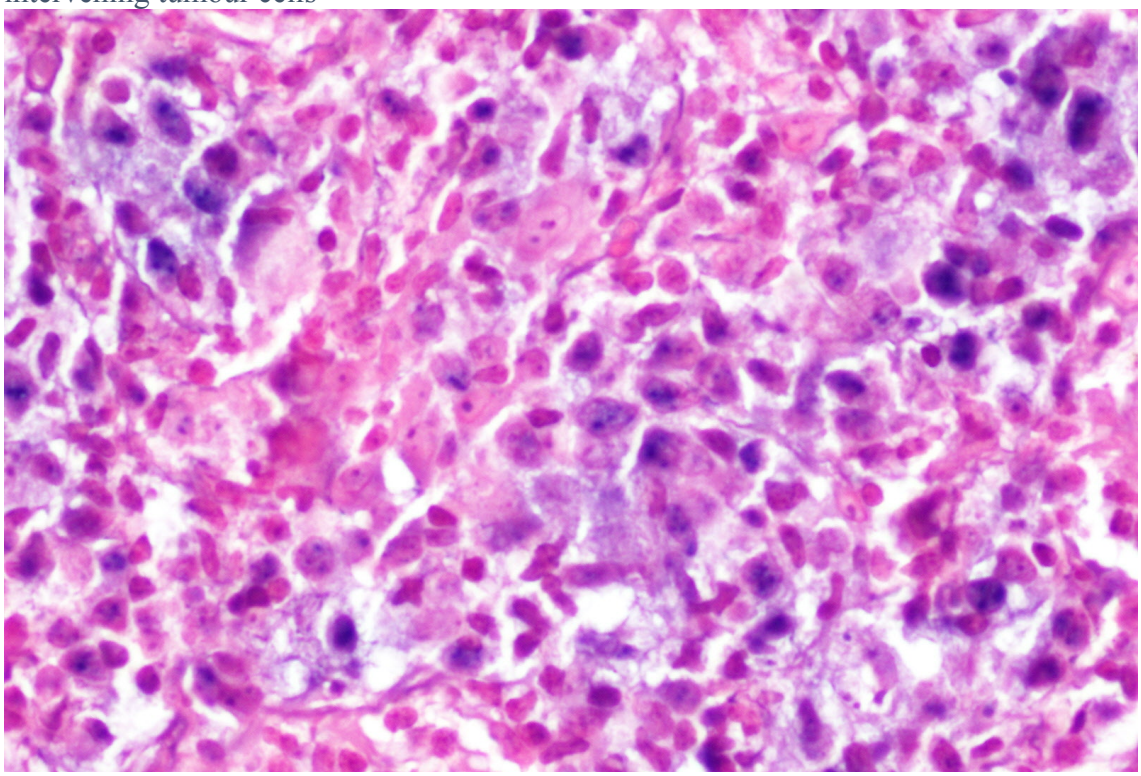
Photomicrograph 23: EBER-ISH, 400X-EBER-ISH positive- Intense blue staining of positive cells on a red background



Photomicrograph 24: EBER-ISH, 400X-Artifactual dull bluish staining of cells, negative for EBER-ISH



Photomicrograph 25: EBER-ISH, 40X-Intense edge artifact with a dull bluish staining of intervening tumour cells



Photomicrograph 26: EBER-ISH, 400X-Same case as above showing a dull artifactual staining of cells, negative for EBER-ISH

DISCUSSION

Ours is possibly the first study on EBV+DLBCL in a cohort of patients older than 45 years of age from the Indian subcontinent as per our knowledge after a thorough literature search. The existing literature points to a varying incidence of EBV+DLBCL of the elderly, as well as a varying median age of patients in different geographic locations.(6,8–10,35,50,63,64) The exact reason behind a geographic variation has yet to be elucidated, although socioeconomic status and endemicity of EBV have been proposed as possible mechanisms.

In a study by Arora et al from the same institute, Non Hodgkin lymphomas accounted for 78% of all lymphomas over a 10-year study period, among which B cell Non Hodgkin lymphomas were by far the commoner subgroup. Diffuse Large B cell lymphoma, not otherwise specified, was the commonest type of B cell Non Hodgkin lymphoma, accounting for almost 47% of all Non Hodgkin lymphomas.

Clinical and epidemiological parameters

Frequency of EBV+DLBCL in patients older than 45 years of age:

We calculated a frequency of 6.42% for EBV positive DLBCL in our cohort of patients older than 45 years of age. This was comparable to that reported in patients from Turkey, Mexico and Saudi Arabia.(10,65,66) The frequency in our cohort is higher than that reported in the European countries and one study each from Korea and Japan.(10,15,16,67) On the other hand, a much higher incidence of EBV positive DLBCL has been reported from Japan, Korea and Peru.(6–8,14)

Age of patients

The median age of EBV+DLBCL cases in our study was found to be 63 years with an age range of 50-82 years, and that of the EBV negative group was 61 years with an age range of 47-83 years. This was found to be much lower than that reported by other investigators. A case series from Peru by Beltran et al reported a slightly older median age of 75 years, ranging from 51 to 95 years.(6) A multicenter study in 2012 by Montes-Moreno et al reported a similar slightly higher mean age of 69 years, with a range of 48-91 years.(9) Chi Young Ok and group also reported a higher median age of 71 years in their cohort of patients.(35) An earlier study by Oyama et al from Japan in 2003 reported a median age of 75.5 years and a range of 60-88 years, in a cohort of 22 patients.(50) A lower median age of EBV positive DLBCLs was reported by Hoeller et al from Switzerland, with a median age of 67 years although they included all age groups and not just patients older than 50 years.(16)Hofscheier et al in 2011 compared the geographic prevalence of EBV+DLBCL of the elderly in German and Mexican populations and found a significantly lower median age of 66 years (range 51-75 years) in the Mexican cohort when compared to the German cohort who showed a median age of 77 years (range 63-88 years). They further observed an increased proportion of cases that showed a small population of cells staining positive for EBER (<20%), possibly representing unrelated EBV transformed cells, or a secondary EBV infection of an already established B cell clone.(10) Possibilities for an earlier age of onset of EBV related lymphoma in these populations include a lower socio-economic status with

accompanying lower standards of healthcare, endemicity to EBV and also racial differences. More recently, EBV positive DLBCL has also been demonstrated in immunocompetent patients less than 45 years of age, and these cases have been shown to have a better prognosis.(51,52) Ok et al in 2015 compared cases of EBV+DLBCL in patients more than 50 years of age and less than 50 years of age, and found that both groups had similar clinicopathologic, immunophenotypic and genetic features, and tests on gene expression and microRNA profiling did not reveal and distinction between the two groups. They further suggested that the arbitrary age cut off for EBV+DLBCL as suggested by the WHO was unnecessary, and ought to be eliminated.(53)

Gender

All the cases of EBV+DLBCL in patients older than 45 years were males in our study. The available literature also suggests a male preponderance in these cases similar to our study, although our male: female ratio is much higher than that reported in literature which averages 1.4:1 to 1.5:1.(9,10,16,35,50)

Demographics

Ours is a tertiary care super-specialty hospital, which caters to a large part of the population from Eastern and Southern India, and this has been reflected in

outpatient demographics. We did not observe any specific or significant geographic variation in the EBV positive cases of DLBCL. We had initially expected a slight increased prevalence of EBV+DLBCL in patients from the northeastern states of India, based on previous studies on the geographic variations of nasopharyngeal carcinoma in India and a study on the sero-epidemiology of EBV in Indian population.(68–70) Whether a larger sample size would have demonstrated a geographical variation is not known.

B symptoms

B symptoms were seen in 5 out of 6 cases of EBV positive DLBCL (83%) and in 29 of 70 cases of EBV negative DLBCL (58.5%). This was similar to the higher occurrence of B symptoms at presentation in EBV positive DLBCL as reported by Oyama et al and Beltran et al.(6,8)

Clinical stage at presentation

In our study, the clinical stage was available for 6 of the EBV positive cases, and all of these presented at a higher Ann Arbor stage (III and IV), in comparison to the 55 EBV negative cases of DLBCL of whom 33 presented at a higher clinical stage (60%). Our findings in both groups are relatively higher than those reported by studies from Peru, Germany, Mexico, Japan and the USA among others, although

the finding of a more frequent higher stage at presentation in EBV positive DLBCL is consistent with currently available literature.(6,8–10,64)

IPI score

38 of the total 129 cases had a documented IPI score, 4 of which were EBER-ISH positive cases. 3 (75%) of the EBV positive cases of DLBCL had a high intermediate or more IPI score of $\geq$ 3 in comparison to the EBV negative cases where 21 of 34 cases (61%) showed an IPI score of $\geq$ 3. Oyama et al from Japan reported a similar distribution of cases and found a higher IPI in EBV positive cases when compared with the EBV negative cases of DLBCL, i.e., 54% and 37% respectively.(8) Beltran et al reported IPI scores of >2 in 57% of EBV positive DLBCL in their series from Peru.(6) The general consensus is that of a higher IPI seen in cases of EBV positive DLBCL when compared to the EBV negative counterpart.(9,50,51)

Bone marrow involvement at presentation

92 of the 129 cases in our study had undergone a bone marrow biopsy at the time of diagnosis, including 6 of the EBV positive cases and 86 of the EBV negative cases. Of these, 1 EBV positive case (16%) and 17 EBV negative cases (19%) showed bone marrow involvement by lymphoma. This is in contrast to a series by Beltran et

al who documented extranodal involvement at presentation in 11 of 28 cases (39%) of EBV+DLBCL, of which 2 cases (7%) showed involvement of the bone marrow. A study by Oyama et al in Japan showed extranodal involvement in 33% of EBV+DLBCL and 28% of EBV negative DLBCL, a difference that was not found to be statistically significant.

Histomorphologic analysis

Pattern of infiltrate and cell morphology

A majority of the cases of DLBCL in our study showed a diffuse pattern, i.e., 5 of 7 cases (71.4%) of EBV+DLBCL and 80 of 102 EBV negative DLBCL (78%).A vaguely nodular pattern was evident in 1 case of EBV+DLBCL and 11 cases of EBV negative DLBCL (14.3% and 10.7% respectively). A frank nodular pattern was far less common, being seen in 1 of the EBV positive cases and 3 of the EBV negative cases. These findings were in contrast to those described by Montes-Moreno et al, who found a predominantly nodular to vaguely nodular pattern in a majority of their cases, with the diffuse pattern accounting for a smaller 38% of EBV positive cases.(9)The centroblastic subtype was the commonest morphology seen in cases of DLBCL, including 4 of the 7 EBV positive cases (57.2%) and 83 of 102 EBV negative cases (81.3%) of DLBCL. Two of the seven cases (28.5%) of EBV positive DLBCL showed an increased proportion of immunoblasts, in contrast to the EBV negative group where an increase in immunoblasts was seen in 10 of

102 cases (9.8%). These findings concur with those reported in literature.(6,9,64) The presence of increased number of immunoblasts or cells with plasmacytoid differentiation have been described previously as common features of EBV positive DLBCL and has been said to aid in the morphological identification of EBV positivity in cases of DLBCL.(9,11,50,54,71)

Reactive background

A reactive background was seen in all 7 cases of EBV positive DLBCL, including 1 case that had a marked and prominent background infiltrate of small lymphoid cells and plasma cells. Among the EBV negative cases of DLBCL, 40 cases showed a focal or mild reactive background, including 1 case that had a prominent infiltrate of eosinophils. 52 of the 102 EBV negative cases (51%) showed a readily identifiable reactive background, of which 2 cases showed a marked prominence. Most investigators have variably described the presence of a reactive background as a polymorphous population of lymphocytes. The finding of a prominent reactive background in our study in all EBV positive cases concurs well with the finding of a polymorphous population of cells in most cases of EBV positive DLBCL in other studies.(6,8–10,35,64)

Necrosis

Frank necrosis was found in 2 of 6 EBV positive cases (33.3%) and in 20 of 100 EBV negative cases (20%) of DLBCL in our study. Necrosis could not be assessed in 3 cases. This is similar to the percentage of cases with necrosis seen in a study by Hofscheier et al.(10) Other researchers have, however, reported a larger proportion of cases of EBV positive DLBCL showing necrosis, including many cases with large areas of geographic necrosis.(6,18,63,64,72)

Tingible body macrophages

Tingible body macrophages were present in 5 of 7 cases (71.4%) of EBV positive DLBCL, being only focally seen in one of these cases. The EBV negative DLBCLs showed a lesser proportion of cases with tingible body macrophages, i.e. 53 of 102 cases (51.9%), being only focally present in 26 of these. A variable proportion of histiocytes have been described in a few other series, although a significant difference has not been described between EBV positive and negative DLBCLs.(6,8,12,35,50)

Multinucleate giant cells

Multinucleate giant cells were seen in 6 of the 7 (85.7%) EBV positive DLBCL cases, one of which also showed Hodgkin Reed Sternberg like cells. 47 of the 102

(46%) EBV negative cases showed multinucleate giant cells, 12 of which also showed Hodgkin Reed Sternberg like cells. The presence of multinucleate giant cells, especially with a Reed Sternberg like morphology in cases of EBV positive DLBCL has been described in most other studies. Beltran et al found scattered Reed Sternberg like cells in almost all cases of EBV positive DLBCL in their series.(6) Giant cells including those with a morphologic resemblance to Reed Sternberg cells were found in 15 of 22 cases (68%) of EBV positive cases in a series by Oyama et al from Japan, and in 3 of 9 cases (33%) in a series by Hofscheier et al from Mexico.(10,50) A significant population of multinucleate giant cells with a variable proportion of Reed Sternberg like cells has also been described in a couple of other studies.(9,11,35,63,72)

Vascular proliferation

Vascular proliferation was evident in all 7 cases of EBV positive DLBCL in our study, and in 77 of the EBV negative cases of DLBCL. A further 13 cases of EBV negative DLBCL showed only focal mild features of vascular proliferation. These features however, have not been categorically evaluated in other studies.

Angioinvasion

Angioinvasion was not seen in any of the EBV positive cases of DLBCL in our study, and only seen in a minority of the EBV negative cases (3 of 102).

Angioinvasion per se has not been evaluated in most case series, however angiocentricity and an angiodestructive pattern was described in a few cases in 2 case series by Oyama et al.(8,50)

Fibrosis

In our study, 2 of the 7 EBV positive cases of DLBCL showed a focal mild fibrosis. In contrast, fibrosis was seen in 51 of the 102 EBV negative cases of DLBCL, including 24 cases where it was only focally present. Fibrosis, like vascular proliferation has not been categorically evaluated in available literature. There was no significant association between the degree of intranodal fibrosis and the EBV status.

Perinodal extension

Perinodal extension could not be assessed in 25 cases, including 3 EBV positive cases and 22 negative cases of DLBCL. All the remaining 4 cases of EBV positive DLBCL showed perinodal extension, along with 76 of the remaining 80 cases of EBV negative DLBCL.

Immunohistochemistry:

CD3

Slides of CD3 immunohistochemistry were available in 4 of the EBV positive DLBCL cases and 92 of the EBV negative cases. The percentage of background CD3 positive T cells among the EBV positive cases was <30% in 1 case, 30-60% in 2 cases and >60% in 1 case. Among the 92 EBV negative cases of DLBCL, CD3 positive cells were seen up to 30% in 62 cases, 30-60% in 22 cases and >60% in 8 cases. Although the background CD3 positive T cell population has seldom been quantified or characterized in other studies, this possibly correlates with the prominent inflammatory background that is generally seen in cases of EBV positive DLBCL.(8,50)

CD20

Patterns of CD20 positivity have rarely been studied in cases of EBV positive DLBCL. We found the pattern of CD20 positive cells to reflect the morphologic pattern of the tumour cells as seen on the initial Hematoxylin and Eosin stained sections. There was no statistically significant difference in the pattern of infiltrate between the EBER-ISH positive and EBER-ISH negative cases of DLBCL in our study.

MIB-1 proliferation index

The slides of MIB-1 immunohistochemistry were unavailable in 1 case each of EBV positive and EBV negative DLBCL. The mean MIB-1 proliferation index in EBV positive cases was slightly higher (84.2%, Range 75% - >95%) than the EBV negative cases of DLBCL (80.8%, Range 40% - >95%), although the median MIB-1 index was 85% in both groups. This difference however may not be accurate enough for comparison as we performed an approximate estimation of the MIB-1 index and did not exactly quantify the positive cells. The Ki67 index is generally considered to be high in cases of DLBCL, although a significant difference has not been established in the EBV positive subset of cases.(35) Montes-Moreno et al reported a Ki67 index of >50% in 40/45 (80%) of their cases, while Beltran et al in their study found a median Ki67 of 80%, with a range of 50%-90% among EBV positive DLBCLs.(6,9)

EBV-LMP1 immunohistochemistry

EBV-LMP1 immunohistochemistry slides were available in 7 cases, 3 of which were EBER-ISH positive. One of the EBER-ISH positive cases was negative on EBV-LMP1 immunohistochemistry, while the other two cases showed a focal and a prominent positivity. All the four cases of EBER-ISH negative DLBCL were negative on EBV-LMP1 immunohistochemistry. This disparity in detection of Epstein Barr Virus by EBV-LMP1 immunohistochemistry and EBER in situ

hybridization is known, and described in literature. Montes-Moreno reported EBV-LMP1 positivity in only 37 of 44 (84%) cases which were EBER-ISH positive.(9) Oyama et al found EBV-LMP1 positivity in 67 of the tested 71 cases (94%) in their series form Japan.(8) It is generally regarded that the concordance rate between EBV-LMP1 and EBER-ISH can reach up to 80%-90%, although this figure is not uniformly consistent. In our experience, we found it easier to identify positive cells on an EBER-ISH stained slide when compared to an EBV-LMP1 IHC stained slide, the latter which sometimes showed a patchy or weak cytoplasmic staining.

EBER In Situ Hybridisation

EBER In Situ hybridization could be performed on 114 of the total 129 cases, of which 5 cases showed overwhelming background artifactual staining and hence could not be reliably assessed. 7 cases showed >20% positive tumour cells, 3 of which showed positively staining tumour cells ranging between 50%-80% and 3 cases showed >80% positive tumour cells. 1 case showed 5%-10% positive tumour cells, while 6 other cases showed very occasional positively staining tumour cells. We considered a threshold of 10% or more positive tumour cells for a positive result. All the positive cells could easily be identified at low power examination (100x magnification), owing to the excellent contrast provided by the intense dark blue staining of the positive cells on a red background. We observed focal to diffuse and faint artifactual nuclear staining in a few cases, with the nuclei staining a dull

bluish-red hue. All the cases that showed an artifactual background staining precluding interpretation were core biopsies. We presume that this may be related to formalin fixation of small cores of tissue, as even some of the resected lymph nodes showed a varying degree of edge artifact, i.e. a non-specific diffuse band of staining at the edge of the biopsy.

Sasikala et al from Chennai investigated the "prevalence of Epstein Barr virus infection and its association with p53 expression" in a group of 87 previously untreated Non Hodgkin lymphomas. They reported detection of EBV in11 of 46 cases of DLBCL, which were not restricted to any age group.(73) This apparently higher prevalence of EBV in DLBCL in their series could be due to the detection technique used, i.e. PCR. Polymerase chain reaction has been reported to have a relatively high sensitivity. Lymphomas are generally composed of a highly heterogeneous population that may include non-tumoural EBV infected cells such as EBV positive memory cells or normal non tumour bystander lymphocytes.(74,75)

Immunohistochemistry for EBNA-2 and LMP-1 is easy to use and readily available, although its slightly lower sensitivity compared to other methods, ambiguity in evaluation of the results and inter-observer and intra-observer variability may cause certain issues. The use of a highly sensitive technique such as polymerase chain reaction also requires careful attention to avoid contamination from the saliva of the examiners, as EBV is ubiquitously present, and may lead to false positive results. In situ hybridization, on the other hand, detects EBER-1 that is expressed even during

latent infection, and has the advantage of visualizing a latent infection of EBV in tumour cells on histological sections. Furthermore, owing to the relatively large numbers of EBER-1 copies in the cell, the degeneration of mRNA that occurs during routine formalin fixed paraffin embedded tissue processing is negligible.(34)

EBER-ISH is the currently recommended standard investigation for detection of EBV, for a diagnosis of EBV+DLBCL of the elderly. Although there is no definite consensus on the minimum percentage of positive tumour cells for diagnosis, most available research is based on a cut off value of $\geq$10% or $\geq$20% positive tumour cells(35). This variation in cut off values for EBER-ISH positive cells has been shown to affect the reported disease prevalence by a few researchers.(14,15,67)

The debate over an age threshold for diagnosis of EBV+DLBCL of the elderly

The WHO in 2008 had recognized an entity termed as "EBV positive Diffuse Large B cell lymphoma of the elderly", which was a subset of DLBCLs that were believed to occur in elderly patients who had a defective immune surveillance for Epstein Barr Virus, owing to immune senescence. These cases were known to be resistant to conventional chemotherapy and hence had a worse prognosis. However, in the recent past, there has been an increasing body of research describing a subset of DLBCL cases in younger individuals that show EBV positivity. Hong et al compared EBV positivity in cases of DLBCL from patient >50 years and <50 years, and found a higher prevalence of EBV positivity in the older group. Further,

EBV+DLBCL in the elderly group was more closely associated with unfavorable clinical characteristics such as more advanced stage, higher IPI, two or more sites of extra-nodal involvement and so on. A poor prognostic impact of EBV positivity was also seen only in the elderly group and not in the younger group. There was no significant difference in the prognosis between the EBV positive and the EBV negative groups of DLBCL in younger patients. They suggested the recognition of "EBV positive DLBCL in young adults" as a distinct clinic-pathologic entity.(51)

A study by Ok et al in 2015 evaluated the clinicopathologic, immunophenotypic and genetic features in Caucasian patients with EBV+DLBCL <50 years of age, and compared these to patients who had EBV+DLBCL and were >50 years of age. They also reported that the older patients more often had a poorer performance status. However, both groups were found to have similar clinicopathologic, immunophenotypic and genetic features. Further, gene expression profiling and microRNA profiling of tumours in both groups were not distinct. Based on these findings, they suggested the removal of the arbitrary cut off of 50 years for the definition of EBV+DLBCL.

In view of these recent findings, the 2016 classification of hematopoietic and lymphoid tumours by the WHO has substituted the modifier "elderly" in "EBV positive DLBCL of the elderly" with "not otherwise specified".(5) The designation of NOS emphasizes that there are more specific entities in which EBV positive large B cells can be found, such as in Lymphomatoid granulomatosis.

CONCLUSION

- The frequency of EBV positive Diffuse Large B Cell Lymphoma in patients older than 45 years of age in our setting is 6.42% which is higher than that reported in the European cohort, but much lower than that reported from Japan, Korea and Peru. This is comparable to the reported incidence from Mexico, Turkey and Saudi Arabia.
- The average age of patients with EBV positive DLBCL in our cohort was 65.8 years which is much lower than the global average age of presentation of 71 years. The earlier age of presentation was comparable to a cohort from Mexico with an average age at presentation of 66 years.
- Among the histomorphologic features, the presence of vascular proliferation and increased number of tingible body macrophages were found to be significant features in the EBV positive cases of DLBCL. These features could be used to triage cases of DLBCL for EBER-ISH.
- Edge artifacts and non-specific background staining for EBER-ISH were seen more often in core biopsies, possibly due to problems related to formalin fixation. Whether a tweaking of the fixation time for lymph node core biopsies corrects this issue without affecting other routine histochemical and immunohistochemical stains and molecular pathology work-up, needs to be evaluated.

LIMITATIONS OF THE STUDY

- This study included only nodal cases of Diffuse Large B cell lymphoma, and the calculated frequency reflects only this subset of cases.
- EBV-LMP1 was not available in all cases, hence a comparison with EBER-ISH could not be done.
- There was no follow up of patients for assessment of response to therapy.

ANNEXURES

IRB APPROVAL LETTER

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

January 03, 2015

Dr. Ananth Vikas. J
PG Registrar
Department of Pathology
Christian Medical College, Vellore 632 004

Sub: **Fluid Research Grant Project:**
Epstein Barr Virus related Diffuse large B Cell Lymphoma Of the elderly--Assessment of the frequency in our Setting using the EBER---ISH technique, and comparison Of the morphological features of the EBER positive and EBER negative cases.
Dr. Ananth Vikas. J, Dr. Marie Therese Manipadam, Pathology, Dr. Auro Viswabandya, Clinical Haematology, Mrs. Grace Rebekah, Biostatistics, CMC, Vellore.

Ref: IRB Min No: 9144 [OBSERVE] dated 12.11.2014

CHRISTIAN MEDICAL COLLEGE VELLORE INDIA

Dear Dr. Ananth Vikas. J,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Marie Therese Manipadam, Pathology, CMC, Vellore.

1 of 5

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

DATA COLLECTION PROFORMA

Serial number:

Age: Address (State):

Gender:

Site of lymph node: Bone marrow involvement

B symptoms: Present Absent

Clinical Stage: IPI Score

Histology:

Pattern of infiltrate: Diffuse / Vague Nodular / Others

Subtype: Centroblastic / Immunoblastic / Anaplastic

Reactive background: Absent / Mild or focal / Readily evident

Necrosis: Absent / Focal or individual cell/ Readily evident

Tingible body macrophages: Absent / Few or focal / Readily evident

Multinucleate cells Present Absent

If present, Reed Sternberg type Yes No

Vascular Proliferation Absent / Mild or focal / Readily evident

Angio-invasion Present Absent

Fibrosis Absent / Mild or focal / Readily evident

Perinodal extension Present Absent

Other features if any:

Immunohistochemistry

Markers	CD3	CD20	MIB-1
percentage +ve and pattern if any			

EBV-LMP1 (If available):

EBER-ISH staining: Percentage: Pattern:

PROTOCOL FOR EBER-ISH STAINING ON THE VENTANA BENCHMARK XT PLATFORM

- Warm slide and incubate for 4 minutes at 75^0C
- Apply EZPrep volume adjust and rinse (X 3) and add coverslip
- Warm slide to 76^0C and incubate for 4 minutes
- Rinse and disable heater
- Rinse and adjust slide volume with reaction buffer, apply coverslip
- Warm the slide to 37^0C and incubate for 4 minutes, rinse with reaction buffer
- Add One drop of ISH-Protease 2, apply coverslip and incubate for 8 minutes
- Rinse and adjust slide volume with reaction buffer, apply coverslip
- Apply One drop of INFORM EBER, apply coverslip and incubate for 4 minutes
- Warm slide to 85^0C and incubate for 12 minutes
- Warm slide to 57^0C and incubate for 4 minutes
- Incubate or 1 hour
- Rinse slide with 2X SSC buffer (X 3), and then adjust volume with reaction buffer
- Apply One drop of iVIEW ANTI-FLUOR, apply coverslip and incubate for 20 minutes
- Rinse and adjust volume with reaction buffer (X2)
- Apply One drop of iView blue SA-AP, apply cover slip and incubate 15 minutes
- Rinse slide with reaction buffer

- Apply One drop of iVIEW BLUE ENHAN, incubate for 4 minutes
- Apply one drop of iVIEW BLUE NBT BCIP, incubate 32 minutes
- Rinse and adjust volume with reaction buffer (X2)
- Add one drop of red counterstain, rinse and wash with SSC buffer and reaction buffer

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sl number	demographic	age	sex	site	B symptoms	clinical stage	IPI	marrow involvement	pattern	subtype	reactive background	necrosis	tingible body macrophages	multinucleate giant cells	RS like giant cells	vascular proliferation	angioinvasion	fibrosis	perinodal extension	CD3	CD20	Mib-1	EBV-LMP1	EBER-ISH
1	kerala	59	f	neck	0	IV A	2/5	1	vague nodular	centroblastic	0	0	2	1	1	2	0	0	1	1	diffuse	70-80	-	0
2	west bengal	53	m	level 5 cervical	0	-	-	0	diffuse	centroblastic	1	2	0	1	1	2	0	2	1	1	diffuse	80-90	-	0
3	west bengal	51	m	right inguinal	1	IIIB	-	0	vague nodular, sinusoidal	anaplastic	0	0	0	1	1	2	0	2	1	1	diffuse	70-80	-	0
4	bihar	61	m	para aortic	1	-	-	0	diffuse	centroblastic	0	0	2	0	0	0	0	2	1	1	diffuse	60-70	-	0
5	nepal	25	m	left cervical	0	-	-	0	diffuse	centroblastic	0	2	2	0	0	2	0	2	1	1	diffuse	95	-	0
6	jharkand	55	m	right inguinal	0	III AES	-	0	diffuse	centroblastic	0	0	2	1	0	0	0	0	N/A	1	diffuse	>95	-	0
7	jharkand	56	f	left supraclavicular	1	IBE	-	0	diffuse	centroblastic	2	0	2	0	0	2	0	1	1	1	diffuse	50	-	0
8	kerala	52	m	retroperitoneal	1	IIAX	2/52	0	diffuse	centroblastic	2	0	0	0	0	2	0	2	1	2	diffuse	90	-	0
9	tamil nadu	57	m	left cervical	0	IV	2/5	1	diffuse	anaplastic	1	0	2	1	0	0	0	0	1	1	diffuse	90-60	-	0
10	jharkand	63	m	retroperitoneal	0	-	-	-	diffuse	centroblastic	2	0	0	0	0	2	0	1	1	2	diffuse	70-60	-	0
11	uttarkhand	62	m	right inguinal	-	-	-	0	vague nodular	centroblastic	2	1	0	1	1	1	1	0	1	3	loose aggregates	70-60	-	0
12	tamil nadu	47	m	axillary	1	IIIB	-	0	diffuse	centroblastic	1	0	2	1	0	2	0	1	1	1	diffuse	95	-	0
13	madhya pradesh	58	f	left cervical	0	-	-	-	diffuse+nodular	centroblastic	1	0	0	0	0	2	0	2	minimal	1	diffuse	80	-	0
14	kerala	26	m	left posterior cervical	0	IIIBX	-	0	diffuse	centroblastic	1	0	0	0	0	1	0	1	1	1	diffuse	80	-	0
15	west bengal	66	m	right axillary	0	IIIAE	-	0	diffuse	centroblastic	1	1	0	1	0	0	0	0	1	1	diffuse	95	-	0
16	west bengal	56	f	right axillary	-	-	-	0	diffuse	centroblastic	2	1	2	0	0	1	0	1	1	1	diffuse	90	-	0
17	jharkand	66	m	left submandibular	1	IIIA X	2/5	0	diffuse	centroblastic	2	1	1	0	0	2	0	1	1	1	diffuse	90	-	0
18	tamil nadu	72	f	retroperitoneal	1	-	-	-	diffuse	centroblastic	0	0	2	0	0	2	0	2	1	1	diffuse	>95	-	scant occasional scattered cells
19	west bengal	48	m	left cervical	-	-	-	-	diffuse+nodular	centroblastic	0	2	0	1	0	2	0	2	0	1	diffuse	>90	-	
20	tamil nadu	61	f	left axillary	1	IVB X	4/55	0	diffuse	anaplastic	1	1	1	1	1	2	0	0	N/A	2	diffuse	N/A	occasional positive	0
21	west bengal	61	m	left axillary	1	IVB	5/5	1	diffuse	centroblastic	1	0	1	1	1	1	0	1	N/A	N/A	nodular/scattered	70-80	negative	50-60
22	tamil nadu	55	f	right post auricular	0	IE	-	0	diffuse	centroblastic	2	0	2	1	1	2	0	2	1	3	diffuse	60-70	-	0
23	tamil nadu	72	m	right cervical	1	IIIB	4/5	0	diffuse	centroblastic	1	2	2	1	0	1	0	0	N/A	1	diffuse	>90	-	0
24	west bengal	07	f	left cervical	-	-	-	-	diffuse	centroblastic	1	0	2	1	1	2	0	0	1	1	diffuse	>90	-	0
25	tamil nadu	82	m	right submandibular	0	IVX	3/53	0	diffuse	centroblastic	marked	0	1	0	0	1	0	0	N/A	1	diffuse	>95	-	80-90
26	tamil nadu	71	f	right cervical	0	IIE	2/5	0	diffuse	centroblastic	1	0	2	1	0	2	0	2	1	1	diffuse	90	-	0
27	west bengal	47	m	right cervical	0	-	-	-	vague nodular	anaplastic	1	0	2	1	1	2	0	1	1	1	diffuse	70	-	0

28	assam	64/5	m	left axillary	1	-	-	-	diffuse	centroblastic	1	2	2	0	0	2	0	2	1	1	diffuse	90->9	-	0
29	assam	2	f	left cervical	-	-	-	-	diffuse	centroblastic	1	2	0	1	0	0	1	2	1	1	diffuse	0	negative	0
30	west bengal	6	m	axillary	-	-	-	1	diffuse	centroblastic	1	2	1	0	0	2	0	2	1	2	diffuse	90	-	0
31	tamil nadu	5/6	f	retroperitoneal	1	IVB	2/54/5	1	diffuse	centroblastic	2	1	0	1	0	2	0	1	N/A	2	diffuse	70	-	0
32	bihar	6/35	m	mediastinal	1	IB	5	0	diffuse	centroblastic	1	0	0	0	0	1	1	2	N/A	1	diffuse	90	-	very occasional
33	chattis garh	45	m	right axillary	1	-	-	0	diffuse	centroblastic	2	1	0	1	0	2	0	1	0	2	diffuse	60	-	0
34	-	15	f	left cervical	-	-	-	-	diffuse	centroblastic	1	1	2	1	0	2	0	1	1	3	diffuse	80	negative	0
35	west bengal	2	f	right axillary	1	-	-	0	diffuse	centroblastic	2	2	2	0	0	2	0	1	1	1	diffuse	80	-	0
36	west bengal	50	m	left cervical	1	IIIBX	2/53	0	diffuse	centroblastic, but with many immunoblasts	2	1	0	0	0	2	0	2	1	1	diffuse	90	-	0
37	west bengal	51	m	left cervical	1	IIIBS	5/53	0	diffuse	centroblastic	2	0	0	1	0	2	0	0	1	1	diffuse	90	-	5-10
38	tamil nadu	72	f	left groin	0	IIAX	5/50	-	diffuse	centroblastic with clear cells	2	2	1	0	0	2	0	2	N/A	2	diffuse	80-85	-	0
39	west bengal	52	m	left cervical	1	IIB	5/54	0	diffuse	centroblastic with clear cells	2	1	0	0	0	0	0	2	1	N/A	diffuse	80	-	0
40	jharkand	62	f	left cervical	1	IIIBX	4/5	0	diffuse	centroblastic	2	1	1	1	0	2	0	1	1	1	diffuse	70-80	-	0
41	andhra prades h	63	m	right cervical	-	-	-	-	diffuse	centroblastic	1	2	1	1	0	2	0	0	1	1	diffuse	90-95	-	0
42	nepal	56	m	retroperitoneal	-	-	-	0	diffuse	centroblastic, but with many immunoblasts	2	0	1	1	0	2	0	0	N/A	1	diffuse	80	-	0
43	west bengal	48	f	abdominal	-	IV	-	-	nodular	centroblastic	2 marked	0	0	0	0	1	0	0	N/A	1	nodular	90	-	0
44	andhra prades h	48	f	left inguinal (gen LN pathy)	-	-	-	-	diffuse	centroblastic	1	1	2	1	1	1	0	0	N/A	-	diffuse	70	negative	N/A
45	bangla desh	38	m	cervical	-	-	-	-	diffuse	centroblastic	2	1	1	1	0	2	0	0	1	2	diffuse	90	-	0
46	tamil nadu	61	m	right inguinal (multiple lymph nodes)	0	IVA	3/54	1	diffuse	centroblastic, but with many immunoblasts	2	0	1	1	0	2	0	0	N/A	-	-	70-80	-	0
47	tamil nadu	63	f	diffuse	0	IIIAE	4/51	0	scattered	centroblastic	2	1	1	1	0	2	0	1	1	1	diffuse and scattered	70	-	0
48	tamil nadu	69	f	right submandibular	0	IIA	/5	0	vague nodular	centroblastic	2 marked	0	2	1	0	2	0	0	1	3	diffuse and scattered	80	-	very occasional
49	west bengal	57	m	right iliac	-	-	-	-	diffuse	centroblastic	2	0	0	0	0	0	0	1	N/A	2	diffuse	30-40	-	0
50	west bengal	55	m	left cervical	-	-	-	0	diffuse	centroblastic	1	1	0	0	0	2	0	2	N/A	1	diffuse	60	-	very occasional
51	bangla desh	55	f	left cervical	1	-	-	0	diffuse	centroblastic	1	1	0	0	0	2	0	0	N/A	1	diffuse	>90	-	0
52	west bengal	70/5	m	left axillary	1	IIBEX	4/5	0	diffuse	centroblastic with spindling	1	0	1	1	0	2	0	0	1	1	diffuse	80	-	very occasional
53	manip ur	56	m	?	1	IIBSE	-	0	diffuse	centroblastic	2	0	0	0	0	2	0	0	1	1	diffuse	80	-	0
54	nagala nd	75	m	left supraclavicular	1	IIIB	2/53	0	diffuse	centroblastic with clear cells	1	0	0	1	0	1	0	0	1	-	-	80	positive	80-90
55	kerala	52	m	left cervical	1	IIIBX	/52	0	diffuse	centroblastic	2	2	0	0	0	0	0	0	0	1	-	-	95	-
56	bangla desh	58	f	?	1	IIBE	2/5	0	nodular	centroblastic	2	0	1	1	0	1	0	0	focal	2	nodular	60	-	0

	west	5							vague															>9						
57	bengal	4	m	right axillary	-		-	-	1	nodular	centroblastic		2	0		1	0	0		1	0	0	N/A		3	diffuse	0	-		0	
	west	5																						>9						
58	bengal	9	m	left cervical		0	IV	-	0	diffuse	centroblastic		1	1		0	1	0		2	0	2	N/A		1	diffuse	0	-		0	
										diffuse + vague														N /			80 -			
59	assam	5 4	m	inguinal	-		-	-	0	nodular	centroblastic		2	1		2	1	0		1	0	0		1	A	diffuse	90	-		0	
		6		right																		N/									
60	-	8	f	supraclavicular	-		-	-	-	diffuse	centroblastic		1	0		0	0	0	N/A		0	A		1	1	diffuse	70	-		0	
		8								diffuse+no														N /			>9			
61	assam	0	m	right axillary		1	IIIB	-	0	dular	centroblastic		1	1		1	1	0		1	0	0		1	A	diffuse	0	-		80-90	
								3																			80			
	jharka	6		left inguinal (gen				/																			-				
62	nd	4	m	LN pathy)		0	IIAX	5	0	diffuse	centroblastic		2	0		2	0	0		2	0	1		1	-	diffuse	40	-		0	
								3			centroblastic, focal																80			
		5						/			increase in																-			
63	kerala	1	f	left axillary		1	IVB	5	1	diffuse	immunoblasts		2	1		1	1	0		2	0	0		1	1	diffuse	90	-		0	
		6																									80			
64	assam	8	m	left cervical	-		-	-	-	diffuse	centroblastic		1	0		1	0	0		2	0	0		1	1	diffuse	- 90	-		0	
								3		diffuse +																					
	tamil	5						/		vague																					
65	nadu	9	m	right cervical		1	IVBS	5	0	nodular	centroblastic		2	1		2	1	0		1	0	0		1	1	diffuse	90	-		0	
																											90			
	jharka	6																									-			
66	nd	5	m	right cervical	-		-	-	-	diffuse	centroblastic		1	0		0	0	0		1	0	0		1	2	diffuse	95	-		0	
		5																									80			
67	-	5	m	inguinal	-		-	-	-	diffuse	centroblastic		1	1		0	1	0		2	0	0	N/A		1	diffuse	-	-		N/A	
								4			centroblastic, but with																85			
	jharka	6						/			many immunoblasts																-			
68	nd	1	m	right axillary		1	IIIB	5	0	diffuse	(60%)		2	2		2	1	0		2	0	0	N/A		1	diffuse	90	-		0	
																											85			
	west	5																							N /		-			
69	bengal	0	f	left cervical		0	IIA	0	0	diffuse	centroblastic		2	1		1	0	0		2	0	0		1	A	diffuse	90	-		0	
	tamil	7												N/																	
70	nadu	6	m	cervical	-		-	-	-	diffuse	centroblastic		1	A		0	0	0		2	0	2		1	2	diffuse	80	-		0	
																											85			
	karnata	5												N/													-			
71	ka	4	f	cervical	-		-	-	0	N/A	centroblastic		1	A		0	0	0		0	0	0		1	1	diffuse	90	-		0	
																											90			
	west	5		left																							-			
72	bengal	4	m	submandibular	-		-	-	0	diffuse	centroblastic		2	2		1	1	1		2	0	2		0	1	diffuse	95	-		0	
	andhra										centroblastic, focal																				
	prades	5									increase in																				
73	h	0	f	cervical	-		-	-	-	diffuse	immunoblasts		2	0		1	1	0		2	0	0		1	1	diffuse	80	-		0	
										diffuse +																					
		6								vague	centroblastic, but with																				
74	assam	1	m	right axillary		1	-	-	0	nodular	many immunoblasts		2	0		0	0	0		2	0	0		1	1	diffuse	90	-		0	
	jharka	7									N/a- medium sized																				
75	nd	8	m	left inguinal	-		-	-	-	diffuse	cells	N/a		2		0	0	0		2	0	2		1	-	-	70	-		0	
	tamil	5																													
76	nadu	6	m	right cervical		0	IBEX	-	0	diffuse	centroblastic		1	0		0	0	0		2	0	0		1	-	-	90	-		0	
		6								diffuse+no																	80			
77	-	5	m	?	-		-	-	-	dular	centroblastic		1	0		0	0	0		2	0	0		1	-	-	-	90	-		0	
	west	5																				N/									
78	bengal	2	m	retroperitoneal	-		-	-	-	diffuse	centroblastic		1	2		0	0	0		2	0	A	N/A		1	diffuse	95	-		0	
	west	6																				N/									
79	bengal	6	m	abdominal	-		-	-	-	diffuse	centroblastic		2	0		1	1	0	N/A		0	A		1	2	diffuse	A	-		N/A	
								4			centroblastic, focal																				
	west	7						/			increase in																				
80	bengal	0	f	right cervical		1	IVB	5	0	diffuse	immunoblasts		1	0		1	1	0		1	0	0		1	2	diffuse	80		0	very occasional	
	west	6		right																											
81	bengal	4	m	submandibular	-		-	-	0	diffuse	centroblastic		2	2		1	1	0		2	0	0		1	1	diffuse	70	-		0	
																											40			
	west	5																				N/					-			
82	bengal	4	m	right inguinal	-		-	-	0	diffuse	centroblastic		2	0		0	0	0		2	0	A		1	2	diffuse	50	-		0	
	jharka	6		right posterior																											
83	nd	7	f	cervical	-		-	-	1	diffuse	centroblastic		1	0		0	0	0		2	0	2		1	1	diffuse	60	-		0	
				left																											
	west	6		submandibular																											
84	bengal	1	m	(gen LN pathy)		1	IIB	1	0	diffuse	centroblastic		1	1		0	0	0		0	0	0		1	1	diffuse	70	-		0	
	tamil	5																													
85	nadu	1	f	mediastinal		1	-	-	1	diffuse	centroblastic		2	1		2	0	0		2	0	0		1	1	diffuse	90		0	N/A	

	west	5																						>9			
86	bengal	0	m	left inguinal		0	-	-	0	diffuse	centroblastic		1	1	0	0	0	0	0	0	N/A		1	diffuse	0	-	0
	andhra							3																			
	prades	7		left				/																			
87	h	8	m	supraclavicular		1	IIIB	5	0	diffuse	centroblastic		2	2	2	1	0	0	0	0		1	1	diffuse	90	-	-
	tamil	5																						>9			
88	nadu	6	f	right cervical		0	IAE	-	0	diffuse	centroblastic		1	0	1	0	0	1	0	0	N/A		1	diffuse	5	-	-
	jharka	5																									
89	nd	7	f	left cervical		0	IVA	-	1	diffuse	centroblastic		1	2	1	0	0	1	0	0		1	1	diffuse	90	-	-
	west	7																									
90	bengal	6	m	para-aortic	-		-	-	0	diffuse	centroblastic		0	0	0	0	0	2	0	2	N/A		2	diffuse	90	-	-
	andhra							5																			
	prades	6						/		diffuse+no														>9			
91	h	0	m	right inguinal		0	IV	5	-	dular	centroblastic		2	2	0	1	0	1	0	1		0	1	diffuse	0	-	-
	west	5								vague																	
92	bengal	0	m	right cervical	-		-	-	1	nodular	centroblastic		1	0	1	1	1	1	0	0		1	1	diffuse	70	-	-
	tamil	5		right																				>9			
93	nadu	7	m	supraclavicular	-		-	-	1	nodular	centroblastic		2	1	0	0	0	2	0	0		1	1	diffuse	5	-	-
																								85			
	west	7																						-			
94	bengal	1	m	left cervical	-		-	-	0	diffuse	centroblastic		1	2	0	1	0	2	0	2	N/A		1	diffuse	90	-	-
	bangla	5																									
95	desh	3	m	right inguinal	-		-	-	0	diffuse	centroblastic		1	1	1	0	0	2	0	1		1	1	diffuse	90	-	-
	andhra									diffuse															85		
	prades	4		left						with	centroblastic with													-			
96	h	8	m	supraclavicular	-		-	-	-	sclerosis	clear cells	N/a		0	0	0	0	2	0	2		1	1	diffuse???	90	-	-
		6									centroblastic, but with																
97	-	7	m	left inguinal	-		-	-	-	diffuse	many immunoblasts		2	1	2	0	0	2	0	1		1	2	diffuse	70	-	-
	tamil	5		left																							
98	nadu	6	m	supraclavicular	-		-	-	0	nodular	centroblastic		1	0	1	0	0	2	0	2		1	1	nodular patchy	90	-	-
																									70		
	west	5																						-			
99	bengal	0	f	right axillary		0	IIAE	-	0	diffuse	centroblastic		2	0	0	0	0	1	0	1		1	2	nodular	80	-	0
	tamil	5		left						diffuse+no																	
100	nadu	7	f	supraclavicular	-		-	-	0	dular	centroblastic		2	0	0	1	0	2	0	2		1	2	diffuse	70	-	0
	west	6																									
101	bengal	2	m	left cervical	-		-	-	-	diffuse	centroblastic		2	0	0	0	0	2	N/A		1		1	2	diffuse	80	-	0
	andhra							2																			
	prades	5						/																>9			
102	h	0	f	right axillary		1	IVB	5	0	diffuse	centroblastic		2	1	2	0	0	0	0	0	N/A		2	diffuse	0	-	-
												1 with															
	tamil	4										eosinoph															
103	nadu	8	m	left axillary	-		III	-	0	diffuse	centroblastic	ils		1	0	1	0	2	0	0		1	3	diffuse	70	-	-
		5					IIBS																				
104	assam	1	m	right inguinal		1	X	-	0	diffuse	centroblastic	N/A		0	1	0	0	2	0	2	N/A		1	diffuse	70	-	-
								3																	50		
	tamil	6						/																	-		
105	nadu	2	f	retroperitoneal		0	IAEx	5	0	diffuse	centroblastic		2	1	0	0	0	0	0	1	N/A		1	diffuse	60	-	0
	jharka	5																									
106	nd	8	m	right cervical		1	IIIB	-	0	diffuse	centroblastic		2	2	2	1	0	2	0	0		1	1	diffuse	90	-	0
																									60		
	tamil	6		left upper deep						diffuse+no															-		
107	nadu	8	m	cervical		0	IIAE	-	0	dular	centroblastic		2	1	0	0	0	2	0	2		1	3	vague nodular	70	? Focal	0
	west	4								diffuse+no	centroblastic with														>9		
108	bengal	9	m	right inguinal	-		-	-	1	dular	clear cells		2	0	2	0	0	2	0	1		1	2	diffuse	0	-	0
	tamil	8																									
109	nadu	0	m	inguinal		1	-	-	0	diffuse	centroblastic		1	0	1	1	0	2	0	1		1	1	diffuse	90	-	0
																									60		
	west	7																						-			
110	bengal	5	m	right cervical	-		-	-	-	diffuse	centroblastic		1	0	0	0	0	2	0	2	N/A		1	diffuse	70	-	0
	tamil	5												N/													
111	nadu	0	m	right axillary	-		-	-	-	diffuse	centroblastic		1	A	0	1	0	1	0	1		1	2	patchy	90	-	60-80
								-		diffuse +														loose	70		
		6						5																aggregates and	-		
112	kerala	3	m	left inguinal		1	IVB	/	0	nodular	centroblastic, but with many immunoblasts		1	1	1	1	0	1	0	0	N/A		2	single cells	80	focal	20-30
	bangla	5						5		vague															>9		
113	desh	7	m	right cervical	-		-	-	-	nodular	centroblastic		2	0	1	1	1	2	0	1	N/A		1	diffuse	5	-	0
																									70		
		7		cervical																					-		
114	-	1	f	(multiple LN)	-		-	-	-	diffuse	centroblastic		2	0	1	1	0	2	0	0		1	2	diffuse	80	-	0
																									80		
	meghal	5																							-		
115	aya	6	f	cervical		1	IVB	-	1	diffuse	centroblastic		2	1	1	1	1	2	0	0		1	3	diffuse	90	-	0

	west	6							diffuse + vague																		
116	bengal	0	m	left axillary	-		-	-	-	nodular	centroblastic	1	1	0	0	0	1	0	1		1	2	vague nodular	90	-		0
	tamil	7						2 /																>9			
117	nadu	5	m	retroperitoneal		0	II	5	0	diffuse	centroblastic	1	0	0	0	0	2	0	2	N/A		1	diffuse	5	-		0
	west	5																									
118	bengal	3	f	right cervical		0	IA	-	0	diffuse	centroblastic	0	2	1	1	0	2	0	0		1	1	diffuse	80	-		0
	tamil	6		left axillary				4 /																>9			
119	nadu	3	m	(with gen LN)		1	IIIBX	5	0	diffuse	centroblastic	1	0	2	0	0	2	0	0		1	1	diffuse	0	-		0
	bangla	5						3 /																80 -			
120	desh	0	m	right inguinal		1	IVB	5	1	diffuse	centroblastic	1	0	0	0	0	2	0	0	N/A		1	diffuse	90	-		0
	west	7		left																							
121	bengal	2	m	supraclavicular		1	-	-	-	diffuse	centroblastic	1	0	0	0	0	2	0	0		0	1	diffuse	95		0	N/A
	west	4																									
122	bengal	8	f	left cervical	-		-	-	-	diffuse	centroblastic	1	2	0	0	0	2	0	1		1	1	diffuse	70	-		0
	tamil	6																						90 -			
123	nadu	5	f	axillary		1	-	-	1	diffuse	centroblastic	2	0	2	0	0	2	0	0		1	2	diffuse	95	-		0
	west	7																						80 -			
124	bengal	2	m	right inguinal	-		-	-	0	diffuse	centroblastic	2	2	2	0	0	2	0	0		0	1	diffuse	90	-		0
		5																				N /		80 -			
125	assam	9	f	left cervical	-		IIIE	-	0	diffuse	centroblastic	1	0	0	0	0	2	0	0	minimal		A	diffuse	90	-		0
	jharka	5									centroblastic, but with													N/			
126	nd	0	m	right inguinal		1	IIIB	-	0	diffuse	many immunoblasts	1	0	1	1	0	1	0	0		1	3	diffuse	A	-		50-60
		7									centroblastic, but with																
127	kerala	0	f	axillary	-		-	-	1	diffuse	many immunoblasts	2	1	1	1	0	2	0	0		1	1	diffuse	80	-		0
	west	6																						>9			
128	bengal	2	m	left axillary		1	-	-	-	diffuse	centroblastic	2	1	0	0	0	2	0	0		1	2	diffuse	5	-		0
	west	6									centroblastic, but with												non uniform				
129	bengal	7	m	right cervical	-		-	-	0	nodular	many immunoblasts	2	0	0	1	1	2	0	1		1	3	staining	80	-		0